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Thermodynamic Analysis of Muscle ATPase Mechanisms

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I. INTRODUCTION

Adenosine triphosphatases play the central roles in physiological functions of cells as energy transducers by coupling the chemical reactions of ATP hydrolysis to the other energetic processes. This review concentrates on the two major ATPases that are characteristic of muscle: actomyosin ATPase of the contractile machinery and the Ca^{2+}-pump ATPase of the sarcoplasmic reticulum (SR). In each case the catalytic cycle of ATP hydrolysis consists of a series of intermediate states of the ATPase molecules. Current research is focused on the correlation of such biochemical mechanisms with physical events resulting in the development of mechanical work in muscle contraction or production of work required for translocation of Ca^{2+} across the SR membrane against the chemical potential of the cations. My main concern is the thermodynamic characterization of the intermediate steps of the ATPase cycle, i.e., the evaluation of the thermodynamic parameters associated with these steps: changes in free energy (ΔG), enthalpy or heat (ΔH), entropy (ΔS), and heat capacity at constant pressure (ΔC_p).

There are two major goals of such thermodynamic studies. First, evaluation of the free-energy changes for transitions between different intermediate states provides an energetic basis to correlate intermediates of the enzyme in a catalytic cycle with its physical states in doing work. This goal is to ultimately identify the most crucial step or steps in the energy-transduction process. The problem in the actomyosin ATPase, for example, is to decide which transition between the intermediates is energetically feasible for force generation in muscle contraction. Considerable progress has been made during the last 10 years owing to the extensive kinetic studies supported by a wide range of new analytical techniques. Indeed it is now possible to depict a reasonable free-energy profile for the actomyosin ATPase in solution. The next step is to extend such in vitro analysis to the actomyosin system in a more organized structure so that the coupling of chemical and mechanical phenomena can be investigated. There have already been several studies in which serious efforts were made in this direction (46, 97, 189, 212).

In the case of the Ca^{2+}-pump ATPase, the relative free-energy levels have not been evaluated unequivocally for all of the major intermediate states. However, investigation of the Ca^{2+} pump has the advantage that its usual experimental system in vitro, the SR vesicles prepared from muscle homogenate, can perform the coupling of chemical work and ion transport. In addition the ATPase alters its affinity for Ca^{2+} when facing opposite sides of the membrane during its catalytic cycle; this is thought to be the most fundamental feature shared by active transport of ions across a membrane in general. Thus the basic problem of this ATPase reaction is well defined,

and intermediate states can reasonably be assigned to those having different directions and affinities in Ca^{2+} binding. Thermodynamic refinement of such a mechanism of the chemiosmotic energy transduction is now in progress.

Second, from the assessment of enthalpy and entropy contributions to the free-energy change, it is possible to have an insight into the nature of a given chemical reaction. The signs and magnitudes of enthalpy and entropy changes are good measures of the nature of the various forces, their relative strength, and their importance in the reaction. If the heat-capacity change is also evaluated, the insight is deepened, as described in this review. This kind of information, brought by thermodynamics, is difficult to obtain by other methods. In addition it is complementary to that obtained by structural and spectroscopic studies. X-ray diffraction, electron microscopy, and other related techniques are essential to describe the gross morphological changes accompanying the energy-transduction processes, particularly in muscle contraction. A wide range of spectroscopic methods probes the microenvironment of a specific residue or residues of ATPase proteins; hence, in combination with kinetic methods, they are also essential to identify and characterize the intermediate states of the ATPase reaction cycle.

In this review, a description is given of the methods for experimental evaluation of the major thermodynamic parameters of the intermediate steps of the ATPase reactions. I emphasize calorimetry, because instrumental improvements recently have been made enabling one to apply it to the two muscle ATPase systems. Because the kinetic knowledge and related information about these systems are essential for the calorimetric analysis as well as thermodynamic discussion, recent results are also summarized.

I discuss implications of the results obtained by these methods in relation to the mechanisms of the ATP hydrolysis coupled with other energetic processes. Based on these results, I discuss certain general features of chemomechanical and chemiosmotic energy transductions.

Even if the range of topics to be covered is confined as described above, the scope of the thermodynamics of biological energy transduction is enormous and requires a vast accumulation of knowledge and information obtained from various types of studies. However, this review is by no means intended to be a comprehensive coverage of the literature on such studies. Hence, taking advantage of the fact that several reviews concerning the energy-transducing ATPase systems are published every year (for a very recent, comprehensive collection of reviews see ref. 219), only a limited number of original papers published earlier than 1977 are cited.

II. METHODS OF THERMODYNAMIC ANALYSIS

A. Thermodynamic Quantities

Principal thermodynamic parameters and their interrelations are briefly summarized here.[1] For a more formal treatment of biothermodynamics and

[1] 1 cal = 4.184 J.

related physicochemical subjects, readers are referred to many excellent textbooks (e.g., refs. 68, 152, 198, 211). A recent book written by Edsall and Gutfreund (66) is especially concerned with analysis and interpretation of data obtained from biochemical reactions, ligand binding, and calorimetric measurements of biological systems.

1. Free-energy changes

The Gibbs free-energy change (ΔG) is a fundamental criterion for the direction and extent of a chemical process, and the magnitude of its decrease ($-\Delta G$) is the direct measure of the maximum capacity of the process to transfer energy as useful work at constant temperature and pressure. For a given chemical reaction, which is held away from equilibrium, ΔG is given by

$$\Delta G = -RT \ln (K/Q) \qquad (1)$$

where R is the gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$), T is the thermodynamic temperature in Kelvin scale, K is the equilibrium constant that equals the mass-action ratio of concentrations of the products to those of the reactants at equilibrium,[2] and Q is the observed mass-action ratio relating to the concentrations when the reaction is displaced from equilbrium. Hence ΔG, referred to as the actual free-energy change, has a value that is a function of the displacement from equilibrium. The standard Gibbs free energy (ΔG^0) is defined as the value of ΔG when the value of Q is unity. Thus by definition

$$\Delta G^0 = -RT \ln K \qquad (2)$$

By substituting this relationship into *Equation 1*, the actual and standard free-energy changes are related

$$\Delta G = \Delta G^0 + RT \ln Q$$

Consequently the actual free-energy change can be calculated for any reaction if the concentrations of the reactants and products are analytically determined, provided the appropriate value of ΔG^0 is known for the conditions (pH, ionic strength, temperature, etc.) under which the reaction will occur. Hence the first step of thermodynamic analysis is to evaluate ΔG^0 by determining K.

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[2] Strictly speaking, thermodynamic discussion should be in terms of activities instead of concentrations of reactants and products. However, there may be no serious consequence in the use of concentration as long as both Q and K for the reaction are evaluated under comparable conditions.

2. Basic free-energy changes

Both the actual and standard free energy refer to the system as a whole. In considering the bioenergetics, particularly the energy-transducing processes, it is often necessary to define the relative free-energy levels of the intermediates of the reaction. These intermediates are different states of an individual (time-averaged) macromolecule, whose free-energy levels are referred to as the "basic" free-energy levels by Simmons and Hill (253). This concept is useful to describe the energy transduction of biological processes, muscle contraction in particular (73, 74).

Consider, as an example, the reaction

$$M + N \rightleftharpoons M \cdot N$$

where M is a macromolecule (e.g., myosin) and N is a ligand (e.g., ADP), which form a complex M · N (see sects. III*C1* and VI*C2*). Let k_f and k_b be the forward and backward rate constants, respectively. k_f is the second-order rate constant with the unit $M^{-1}t^{-1}$, and k_b is the first-order constant with the unit t^{-1}, where t is an appropriate unit of time. Because at equilibrium the rates will be equal for both directions, employing equilibrium concentrations (denoted by subscript e)

$$k_f(M)_e(N)_e = k_b(M \cdot N)_e$$

and

$$\frac{(M \cdot N)_e}{(M)_e(N)_e} = k_f/k_b = K$$

By rearranging this equation, one obtains

$$K \cdot (N)_e = (M \cdot N)_e/(M)_e$$

which is the equilibrium ratio of probabilities of the two states of the macromolecule, M · N (complexed) and M (free), at a given (equilibrium) concentration of the ligand. This ratio is called the basic or effective equilibrium constant, K_{basic}. Thus

$$K_{basic} = (M \cdot N)_e/(M)_e$$

and the basic free-energy change (ΔG_{basic}) for the reaction is

$$\Delta G_{basic} = -RT \ln K_{basic}$$

Note also the relationship between K_{basic} and the rate constants

$$K_{basic} = k_f(N)_e/k_b$$

Because $k_f(N)_e$ has the unit t^{-1}, it is referred to as the pseudo-first-order rate constant. In other words, the basic equilibrium constant is the ratio of the first-order rate constants for both directions; hence the basic free energy is directly related to the rate of the process.

As is obvious from the definition, the actual concentration of the ligand is taken into account for ΔG_{basic}. This fact is relevant to the argument in terms of the drive of the energy-transducing systems in which the concentrations of ligands involved (ATP as reactant and ADP and P_i as products) are kept rather constant, as in the muscle cell. Incidentally, for reactions not involving the binding or release of a ligand such as isomerization of a macromolecule, the basic free-energy change is numerically equal to the standard free-energy change.

3. Enthalpy and entropy changes

The energy-entropy relation for changes at constant temperature and pressure is

$$\Delta G = \Delta H - T\Delta S \qquad (3)$$

where ΔH and ΔS are the changes in enthalpy and entropy, respectively. If ΔH can be evaluated, it becomes possible to make the assessment of enthalpic and entropic contributions to the free-energy change. The value of ΔS is calculated with a rearranged form of *Equation 3*

$$\Delta S = (\Delta H - \Delta G)/T$$

For most biochemical reactions that take place in solution, the volume changes (ΔV) are negligible. The ΔH is identical with energy change (ΔE), as is evident from the equation defining ΔH

$$\Delta H = \Delta E + P\Delta V$$

where P is the pressure on the system. Also by definition, ΔH equals the heat change (reaction heat) accompanying a reaction at constant pressure. ΔH is negative for an exothermic reaction (heat lost from the system) and positive for an endothermic reaction (heat gained by the system).[3]

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[3] The sign convention to be used in this review is the same as that used by Wilkie (303) and Curtin and Woledge (47). Heat produced by the system is considered to have a positive sign and, by definition, ΔH values have a negative sign for reactions that produce heat. Therefore the heat produced equals $-\Delta H$ for any reactions when no external work is done.

4. Unitary quantities of thermodynamic parameters

In the thermodynamic arguments of a reaction occurring in a solution, it is often necessary to exclude a complication that arises from changes in the number of solute particles in the reaction. In other words, a thermodynamic parameter is composed of two distinct terms, the *unitary* and the *cratic* (66, 109, 150). The unitary term is fundamentally relevant to the nature of the reaction, whereas the cratic term is only related to the mixing of solute molecules with solvent, hence independent of the nature of the solutes. For reactions in aqueous solution such as the binding of a nucleotide to myosin (see sects. III*C1* and VI*C2*)

$$M + N \rightleftharpoons M \cdot N$$

the cratic term of the entropy change (ΔS_{cratic}) is

$$\Delta S_{cratic} = R \ln X$$

where X is the change in the total mole fraction of solutes. For the standard state of 1 M, X is numerically equal to the reciprocal of the molar concentration of water (1/55.5) in diluted aqueous solution, and the value of ΔS_{cratic} is -33.4 $J \cdot K^{-1} \cdot M^{-1}$. Hence ΔS_u is calculated by subtracting this cratic contribution from the value of the standard entropy change (ΔS^0)

$$\Delta S_u = \Delta S^0 - \Delta S_{cratic}$$

The unitary free-energy change (ΔG_u) can then be calculated on the basis of a reasonable assumption that observed ΔH is a unitary quantity. For the reaction described, the value of ΔG_u is more negative by $\sim$10 kJ/M than ΔG^0 for the standard state of 1 M at $\sim$20°C.

5. Detailed balance of thermodynamic parameters

For a cyclic process consisting of n steps of reactions, if the equilibrium constant for the ith step is defined as K_i, referring to the clockwise direction, the product of all n equilibrium constants is equal to unity. Thus

$$K_1 K_2 \ldots K_n = 1$$

This is called the detailed balance of equilibrium constants in the closed cyclic process (66). From *Equation 2* it is evident that for the free-energy change (ΔG_i^0) for the same cyclic process

$$\sum \Delta G_i^0 = 0$$

Likewise the sums of the enthalpies, entropies, and heat capacities defined in the same way as ΔG_i^0 are numerically nil. These relations are useful because thermodynamic parameters for one of the steps in a cyclic process can be evaluated if the others are known. Examples are found in sections VI and VII.

B. Kinetics and Equilibrium Analysis

In principle it is possible to obtain all the information about energetics for a given reaction from kinetic analysis, because it reveals the rates and hence the equilibrium constant. However, all the necessary kinetic data are rarely obtained, particularly for complex reactions consisting of a sequence of more than two steps. In some cases, equilibrium analysis of partial reactions in multistep reactions provides invaluable data that complement kinetic information (110).

If the equilibrium constant is measured over a range of temperatures and ln K is plotted against $1/T$, ΔH can be estimated by the van't Hoff equation for the variation of the equilibrium constant with temperature[4]

$$\mathrm{d}(\ln K)/\mathrm{d}(1/T) = -\Delta H/R$$

In many cases, however, the slope is not linear, because ΔH also varies with temperature. This tendency is quantitatively expressed by the change in heat capacity (ΔC_p)

$$\Delta C_p = \mathrm{d}(\Delta H)/\mathrm{d}T$$

In theory the magnitude of ΔC_p can be estimated from the degree of curvature in the van't Hoff plot based on data of sufficient precision. Thus measurement of the equilibrium constant for a given reaction by kinetic and equilibrium methods over a temperature range could provide the data necessary to evaluate all principal thermodynamic parameters for the reaction.

However, there is a major practical difficulty in this type of approach. In most cases, values of K, whether estimated by kinetic or equilibrium analysis, are not precise, and it is fair to say that the errors accompanying these estimates usually exceed ±10%. Thus the evaluation of ΔH from the temperature dependence of K with van't Hoff plots may have a large error, which is often not properly appreciated. It is still harder to estimate ΔC_p from the curvature of the van't Hoff plots.

In studying the reactions consisting of a series of intermediate reactions

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[4] To a good first approximation, observed ΔH is independent of concentration of reactants, hence equated with ΔH_u. Also note that, in the strict sense, the van't Hoff equation is based on a two-state model of equilibrium and will not be applicable to a highly cooperative and/or multistate process (239).

such as the ATP hydrolysis by myosin, actomyosin, and Ca^{2+}-pump ATPases, the values of ΔG^0 are estimated by the kinetic methods in combination with the equilibrium analysis as outlined in sections VI and IX. The direct calorimetric measurement of ΔH, known as reaction calorimetry (sect. III), should be used to evaluate the contributions of ΔH^0 and ΔS^0 to ΔG^0 for these reactions.

III. REACTION CALORIMETRY

A. *Introduction*

Calorimetry is a thermodynamic method used to measure heat quantities or heat effects. It may be divided into reaction calorimetry and differential temperature-scanning calorimetry. The former is the measurement of heat change accompanying a given chemical or physical process initiated by mixing two or more reactive components in the calorimeter at constant temperature. The latter is the measurement of the heat capacity of a substance, be it solution or solid, and its variation with temperature. It provides useful information about the thermodynamic principles of organization and stabilization of the unique three-dimensional structure of biological macromolecules (163, 227, 228).

B. *Instrumentation*

1. *General consideration*

There are several calorimetric principles and a large variety of different practical designs suitable for reaction calorimetry. Many of these are described by Brown (32) and in several reviews (167, 256, 257, 259, 285). Some of these instruments are commercially available and have been widely used to study the interaction of proteins and nucleic acids with their ligands (16, 25, 70, 236, 285).

Calorimetric instruments are classified into two types, depending on how the reacting components are mixed to initiate the reaction: the batch calorimeter and flow calorimeter. In a batch calorimeter the two reactant solutions are first somehow separated in the calorimeter cell and mixed after thermal equilibration, then the accompanying heat effect is measured. A high sensitivity is the major advantage of this type of instrument. Although practical sensitivity is limited by various factors not inherent to the instruments, a heat change as small as 0.05–0.1 mJ produced in 1 ml of reaction mixture can be measured at best in some cases. Hence <1 μmol of reactant is sufficient for a single experiment, provided that the enthalpy change exceeds 10 kJ/mol (256).

For thermodynamic analysis in general, however, a set of data obtained by repetition of such experiments is required. The whole experiment would then extend for several days because the thermal equilibration usually takes several hours for each calorimetric run, whereas the heat measurement itself takes little time compared with that for the equilibration.

On the other hand, in a flow calorimeter the reactant solutions are pumped into the calorimeter cell where they are mixed, and a steady-state heat effect is observed that greatly facilitates the accumulation of data. However, this method requires much more material and is less sensitive than batch calorimetry.

2. Calorimetric titration

A titration device was developed by Woledge (304) to speed up the experiment with a batch calorimeter without sacrificing its high sensitivity. This device adds one reactant to the other in the calorimeter cell. This improvement has markedly reduced the time required to study the process of protein saturation with a specific ligand in a number of steps. Thus data for determination of a binding curve (the equilibrium constant, enthalpy change, and stoichiometric relationship between the reactants) can be obtained by a single calorimetric run. Furthermore, if such a calorimetric titration is repeated over a range of temperatures at an appropriate interval, not only can the heat-capacity change (ΔC_p) be evaluated, i.e., the temperature dependence of ΔH, but so can that of C_p and the equilibrium constant (see, e.g., refs. 154, 159, 308).

3. Rapid-response calorimeters

To estimate individual reaction heats for the intermediate steps of the ATPase reaction, such as myosin ATP hydrolysis, it is necessary to follow the time course of heat production immediately after initiation of the reaction. This demands a rapid-response calorimeter with high sensitivity. There have been several designs of fast calorimeters, and the continuous rapid-flow calorimeter developed by Hartridge and Roughton in 1923 (24, 240) should be noted. Although a time resolution in the millisecond range can be obtained, it suffers from the disadvantage that large volumes of solution are needed. Some recent stopped-flow calorimeters have time resolutions of 1 s to 1 ms (15, 24, 29, 213), but the sensitivity is inadequate by at least one order of magnitude.

A rapid-response calorimeter with a compromise of the response and sensitivity was recently constructed (160, 305). In this instrument the thermocouple detectors connected in series provide a sufficient sensitivity to measure heat effects as small as 0.2 mJ. In addition, they are placed so they make a close contact with the reactant solution to minimize heat-conduction

delay. Thus the response is 90% complete in 1.5 s. Although this calorimeter is not as fast as the stopped-flow type, it has been successfully used for the analysis of ATP hydrolysis by myosin and Ca^{2+}-pump ATPase. In addition this instrument has proved to be suitable for calorimetric titration (154). Apart from time required for thermal equilibration after loading the sample, a whole titration process can be completed within several minutes, compared to several hours required in earlier studies (158, 159).

C. Data Processing in Calorimetry

Biltonen and Langerman (25) gave the subject more general treatment. The two most basic points in calorimetric experiments are mentioned here. First, the heat observed in a calorimeter is a complex effect composed of a genuine heat effect (h) accompanying the reaction under investigation and that from several nonspecific sources. Second, the value of h is proportional to the extent of the reaction (ξ) in the calorimeter, so independent analytical experiments are usually necessary to estimate the ξ values.

1. Heat of mixing

Suppose a reaction started in a calorimeter by mixing solutions 1 and 2, which contain reactants A and B, respectively (A and B react to form C). The observed heat effect (h_{obs}) equals the sum of the heat due to solution mixing (h_{mix}) and the heat effect (h_{app}) accompanying the reaction. The h_{app} includes the heat of ionization of the buffer compound used (this problem is discussed in sect. *C3*).

Strictly speaking, h_{mix} consists of two components, the heat due to dilution of reactants and the heat dissipated by any physical processes accompanying the mixing of two solutions and mixing itself. The value of h_{mix} is determined in separate experiments by mixing solution 1 containing A with solution 2 not containing B, or vice versa. However, if appropriate precautions are taken to adjust pH, ionic strength, and composition of two solutions to be mixed, the correction for the h_{mix} will be relatively small.

2. Extent of reaction

The value of h_{app} thus obtained ($h_{app} = h_{obs} - h_{mix}$) is proportional to the amount of C formed (ξ_C), which is related to an apparent enthalpy change of the formation of $C(\Delta H_{app})$ by

$$h_{app} = (-\Delta H_{app}) \cdot \xi_C$$

The determination of ξ_C should be made from a chemical analysis of the reaction mixture after calorimetry. If difficult, the analysis can be made in

separate experiments for the reaction under conditions otherwise the same as calorimetry. In titration calorimetry the problem of determining the extent of the reaction is replaced by the curve-fitting procedure. There is a useful way to control the extent of reaction in some special cases. If the binding constant is known to be very large and the reaction is carried out in a calorimeter with one of the reactants in excess of the other, the reaction of the latter can be regarded as complete. The results can be analyzed by assuming that the extent of reaction equals the initial concentration of reactant not present in excess. An example of this is found in the analysis of myosin/ATP interaction (see sect. VII*C*).

3. Heat of buffer ionization

In many reactions involving biological macromolecules, some protons are liberated or absorbed through the shift of pK values of certain ionizing groups. Therefore the heat produced by interaction of these protons with the buffer present in the reaction system is included in the values of ΔH_{app}. There are two methods of correcting for this heat. One is to measure in separate experiments the amount of hydrogen liberated or released (n_H) with a pH electrode and the heat of protonation of the buffer (ΔH_{buffer}). The true enthalpy change for the reaction (ΔH) then will be given by

$$\Delta H = \Delta H_{app} - n_H \Delta H_{buffer}$$

Another method is to repeat the calorimetric experiments with buffers having different protonation heats. Assuming that the n_H value is the same in two different buffers, both ΔH and n_H can be determined. The Ca^{2+}-pump ATPase reaction has been analyzed with this method (sect. IX*E4*).

The protonation heat values of buffers widely used in biological experiments can be found elsewhere (87, 128). The temperature-dependence table of buffer pK values is also useful because the protonation heat can be worked out with the van't Hoff relation (98, 113, 221).

IV. INTERPRETATION OF THERMODYNAMIC DATA

A. Introduction

This subject is discussed in detail by Eftink and Biltonen (70) in a recent, comprehensive review, and the results relevant to my review are briefly summarized.

It is possible to foresee how far a chemical reaction proceeds if the initial concentrations of the reactants and the standard free-energy change of the reaction are known. However, the standard free-energy change value may

be relatively insensitive to variation in the molecular details of the reaction, because the enthalpy (ΔH) and entropy ($T\Delta S$) contributions tend to compensate for one another (70, 174). The situation is often encountered when a protein/ligand interaction is studied as a function of ionic strength, pH, or temperature or with different, but structurally related, ligands. Whatever the actual mechanism of such an "enthalpy-entropy compensation effect" (69), it is of fundamental importance to evaluate the enthalpy and entropy contributions to the free-energy change in thermodynamic analysis.

Current research suggests that the sign and magnitude of thermodynamic parameters constitute very important diagnostic molecular mechanisms of many biochemical processes (70, 239, 260). Several possible inter- and intramolecular effects are summarized in Table 1, some of which are discussed below.

B. Interaction of Water with Biological Molecules

1. Importance of hydration/dehydration

Hydrophobic and electrostatic effects are the two major effects by which the state of water is altered by its interaction with biological molecules, thereby making large contributions to the thermodynamic state of the system. Although these effects are different in nature, both are related to the hydration/dehydration of the solute molecules in water. In an enzymatic process therefore they play important roles in the transfer of reactants from the bulk-water phase to the surface of the enzyme protein, their interaction with specific protein groups at the catalytic site, and concomitant conformational

TABLE 1. *Major sources of thermodynamic changes in reactions involving proteins*

Sources	ΔH	ΔS_u	$\Delta C_{p,u}$
Exposure of nonpolar groups to water	−	−	+
Exposure of charged groups to water	+ or −	−	−
Van der Waals interaction	−	−	−
Formation of H^+ bond	−	−	+
Increase in intramolecular motions of protein	+	+	+
Increase in number of isoenergetic conformations of protein	0	+	0
Shift of equilibrium between macrostates of protein*			

ΔH, change in enthalpy; ΔS_u, change in entropy; $\Delta C_{p,u}$, change in heat capacity. * Signs and magnitudes depend on system (see text). [Based on data from Ross and Subramanian (239) and Sturtevant (260).]

changes of the enzyme proteins that are most likely to change the degree of exposure of charged and/or nonpolar protein groups to water (67).

2. Hydrophobic effect

The importance of this effect in biological processes is now well appreciated, and it can be quantitatively treated in physicochemical terms (21, 96, 269).

Qualitatively the hydrophobic effect refers to the tendency of nonpolar groups in water to associate preferentially with themselves or other nonpolar groups, because "cages" of structured water are formed around nonpolar groups. Similar to its state in ice, water in such a state has a low entropy and a high heat capacity. The association of nonpolar groups in the aqueous environment or the transfer of nonpolar groups from the aqueous to nonaqueous environment is accompanied by the breakdown of the water cages. These processes are therefore energetically favorable ones, characterized by a large positive entropy ($\Delta S \gg 0$) and large negative heat-capacity changes ($\Delta C_p < 0$).

The ΔH for the hydrophobic effect is usually small and often makes a negative contribution to the free-energy change. For example, the transfer of ethane from water to chloroform is accompanied by a slightly unfavorable ΔH of +7.5 kJ/mol, and the process is entropically driven with ΔS of 79 $J \cdot K^{-1} \cdot mol^{-1}$ at 25°C (269). It should be noted, however, that because $\Delta C_p < 0$ in the hydrophobic effect, ΔH becomes more negative with an increase in temperature, making ΔS less positive at higher temperatures. This arises because of the diminishing water structure at higher temperatures.

3. Electrostatic interactions

When charged molecules are introduced into water, several water molecules are attracted, oriented, and compressed by the intense electrostatic field around the charged molecules (see ref. 68, p. 450–457). Those water molecules are in a low entropy state, but their heat capacity is also lowered because their structure is less labile than it would be in pure water (269). The ΔH for the interaction of charged molecules with water shows either a negative or positive sign, but its magnitude is not large as long as no net change occurs in the number or strength of hydrogen bonds. On the basis of the thermodynamic nature of the interaction between charged molecules and water, the strong interaction between a charged molecule and a countercharged molecule in the aqueous environment is driven primarily by an increase in entropy due to the release of water from around the charged molecules. Enthalpy changes (of either sign) are small, and ΔC_p is positive.

A good example illustrating the contribution of the electrostatic effect to the thermodynamic change may be provided by the interaction of divalent

cations such as Mg^{2+} and Ca^{2+} with negatively charged groups of proteins, nucleic acids, and related molecules. As expected from the state of the reacting groups in an aqueous solution in which they are surrounded by water molecules, the binding of Mg^{2+} to ATP, for example, is totally driven by a large positive entropy change that dominates the slightly unfavorable enthalpy change (4).

C. Conformational Changes

1. Unfolding of protein

Of the conformational changes possible for proteins, the most extreme is probably the unfolding accompanying denaturation. This is a transition of the polypeptide chain from its state of a certain compact structure to a state that is close to a random coil. As a result of intensive thermodynamic studies, particularly by Privalov with a scanning-calorimetric technique (227, 228), it is now possible to evaluate thermodynamic parameters for protein unfolding. Roughly speaking, for a typical globular protein with a molecular weight of 25,000, denaturation occurs with a ΔH of 200–400 kJ/mol, ΔS of 0.4–1.5 $kJ \cdot K^{-1} \cdot mol^{-1}$, and ΔC_p of 4–17 $kJ \cdot K^{-1} \cdot mol^{-1}$.

These thermodynamic changes are explained in terms of the changes induced on unfolding. A large ΔH is probably due to the disruption of hydrogen bonds and van der Waals contacts, which contribute to the stabilization of compact structure, whereas a large positive ΔS can largely be attributed to the accompanying increase in configurational freedom of the polypeptide. The most important, if not the only, source of the increase in heat capacity is the interaction of water with nonpolar groups that are exposed on unfolding. Bearing in mind that unfolding is the extreme of protein conformational changes and that it occurs with such large thermodynamic changes, more subtle conformational changes relevant to the enzymatic mechanism are now described.

2. Protein/water interactions followed by intramolecular changes of proteins

As described above, when an enzyme protein interacts with its reactants and goes through different intermediate states in the catalytic process, its hydration should change considerably. For example, if some hydrophobic residues exposed to water become sequestered in the interior hydrophobic regions of the enzyme protein or assembled while remaining on the exterior of the protein (see, e.g., ref. 272), the cages of water around the hydrophobic residues are disrupted. The extent of hydration is also changed in the formation of protein molecules from subunits and in the interaction between

different proteins. When protein surfaces come into close contact with each other, a large number of water molecules are probably set free.

In these cases therefore the primary effect is disordering of the water structure; hence $\Delta S > 0$. If the hydrophobic effect predominated, the sign of ΔC_p would be negative with positive ΔH. However, the observed thermodynamic changes are often hard to explain on this basis alone. In fact, not only ΔC_p but also ΔH and ΔS are predominantly negative in many reactions, particularly ligand binding and self association of proteins (239).

To overcome this difficulty it seems necessary to consider the intramolecular changes of proteins as a consequence of disruption of the hydration layers of proteins and ligands. Sturtevant (260) suggested the importance of the contribution of changes in intramolecular motions of proteins to ΔC_p and ΔS, including vibration and rotation of side chains and backbone bending, which are characterized by force constants weak enough to be readily perturbed by ligand binding or partial unfolding. On the other hand, Ross and Subramanian (239) emphasized that the strengthening of hydrogen bonds in the low-dielectric protein interior and van der Waals interactions (see Table 1) are the most important factors contributing to the observed negative values of ΔH and ΔS. Apart from the large negative ΔC_p, both theories predict that with an increase in temperature, enthalpic contribution (more negative or less positive) becomes dominant in stabilizing a conformation of many proteins (239).

3. Shift in equilibrium between protein macrostates

With the accumulation of data from direct calorimetric measurements on many processes involving proteins, a strong temperature dependence of the heat-capacity change has also been detected (82, 153, 154). This phenomenon is difficult to explain on the basis of the hydrophobic effect or other sources described above, because they are observed within a small temperature range.

If a protein exists in different macroscopic structural states that are distinguished by their peptide-folding patterns in at least part of the structure and the equilibrium between these macrostates is temperature dependent, their redistribution caused by interaction with other molecules could be a source of a large ΔC_p and temperature dependence of ΔC_p (69, 70).

In the case of a protein/ligand interaction, elements of this hypothesis are: *1*) a protein exists in (at least) two macrostates, one of which is in a high-enthalpy state and the other in a low-enthalpy state; *2*) the transition between these states is controlled by a substantial enthalpy change (ΔH_{trans}) and a relatively small equilibrium constant (K_{trans}) that is in unity within or near the experimentally feasible range of temperature; and *3*) the affinity for a ligand is different for the two macrostates. By mathematical formulation

the apparent enthalpy change for the ligand binding (ΔH_{app}) can be shown as a function of the intrinsic heat of the ligand binding (ΔH_{lig}), ΔH_{trans}, K_{trans}, and temperature (69, 70, 82). The apparent heat-capacity change ($\Delta C_{p,app}$) and its temperature dependence are, of course, the first and second temperature derivatives of ΔH_{app}. The most important prediction made by this hypothesis is that even if both ΔH_{trans} and ΔH_{lig} were temperature independent, the ligand binding would apparently be accompanied by a negative heat-capacity change ($\Delta C_{p,app} < 0$) also showing a temperature dependence. The nucleotide binding to myosin is probably best explained by this hypothesis, described in section VIII*C*.

In summary, there are many possible sources for the thermodynamic changes accompanying processes in which proteins play the primary role through the interaction with other molecules. One wishes to relate particular sources to the experimentally obtained thermodynamic parameters. However, because they are the sums of contributions from several different sources, quantitative evaluation is difficult at present. Nonetheless, comparisons of thermodynamic data with information on mechanistic changes obtained by other analytical methods will definitely provide useful insight into these processes involving biological molecules.

V. ENERGETICS OF ATP HYDROLYSIS

A. Introduction

From the kinetic viewpoint ATP is relatively stable under physiological conditions, but it is unstable in the thermodynamic sense that the equilibrium of the hydrolysis reaction

$$ATP + H_2O \rightleftharpoons ADP + P_i$$

lies far to the right. Thus the ATP-ADP system serves as a carrier of chemical energy from metabolic reactions for use in most energy-requiring processes in cells.

Since its discovery, ATP and related compounds have been the subject of extensive studies from various aspects. There are many good articles and reviews about the history of the discovery of ATP and subsequent development of the "high-energy phosphate bond" concept (83), chemical kinetics of polyphosphate hydrolysis (71, 142), quantum chemistry (229), theoretical thermodynamic analysis (94, 95), and calculation methods of thermodynamic parameters for ATP hydrolysis as a function of metal ion concentration and pH (3–5). The results that are relevant to this discussion are briefly summarized.

B. Thermodynamic Analysis of ATP Hydrolysis

1. Equilibrium constant and free-energy change

The thermodynamic analysis of the ATP hydrolysis is complicated for two reasons. First, the reaction involves a variety of molecular species, because all the reactants and products are weak acids having more than two dissociable protons (polybasic acids). They are partially ionized at physiological pH, forming various anionic species. In addition, these anions form complexes with metal ions of different affinities. The complex formation with Mg^{2+} is particularly important because of its high affinity for the ionized forms of these phosphate compounds. It is not negligible when the hydrolysis reaction is studied under physiological conditions for which a relatively high concentration of the cation (0.6–1 mM; ref. 108) is required.

For this complicated situation, the ATP hydrolysis reaction is treated, by convention, as having an apparent equilibrium constant (K_{app}; often as K' or K_{obs} in the literature)

$$K_{app} = [ADP] \cdot [P_i]/[ATP]$$

where the concentrations are those determined analytically and include all ionized and Mg^{2+}-complexed species of each component. In other words, any effects of ionization or chelation are ignored, so a value of K_{app} is only valid for a given pH and $[Mg^{2+}]$. The method of calculating K_{app} based on information about these conditions has been formulated by Alberty (3, 4).

The second difficulty is that it is almost impossible to evaluate the K_{app} and hence the apparent standard free-energy change of ATP hydrolysis in a straightforward fashion, because in the hydrolysis reaction only a trace of ATP would remain at equilibrium. Thus, indirect methods have been used in which two or more reactions are coupled. The net effect is the hydrolysis of ATP together with another reaction, the equilibrium constant of which is exactly known. The glutaminase-glutamine synthetase reactions (22, 238) and acetate kinase-phosphate acetyltransferase reactions (111) have been used so far.

2. Heat of ATP hydrolysis

As for ΔH, direct measurement is possible by calorimetry with an appropriate preparation of ATPase, which should be active under a variety of conditions and behave as a true catalyst that does not bind either reactants or products to an appreciable extent at the end of the reaction.

Myosin was used in earlier studies (151, 225).[5] In fact, myosin shows a

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[5] The ΔH value obtained in these studies (-20.0 ± 0.5 kJ mol^{-1}) at pH 8.0 without Mg^{2+} has been used as the basis in various thermodynamic calculations (*Eqs. 3-5*).

TABLE 2. *Enthalpy change for ATP hydrolysis determined by calorimetry*

Temperature, °C	ΔH/kJ mol^{-1}	
	pH 7.0	pH 8.0
4		16.8*
8	25.2	18.5
12		19.0*
20	26.9	20.3

Values obtained by Kodama et al. (155) with SR vesicles in presence of 4 mM $MgCl_2$ and 6 μM A23187. * With 10 mM $MgCl_2$.

high affinity for ADP and a low catalytic activity (see sect. VI). Recently, Kodama et al. (155) showed that the Ca^{2+}-pump ATPase of SR vesicles is quite useful for the determination of ΔH for the ATP hydrolysis under physiological conditions.[6] Although Ca^{2+}, which is necessary to activate the enzyme, is taken up by the vesicles, there is no net accumulation of the cation in the presence of a Ca ionophore such as A23187 or X537A when ATP hydrolysis has been completed. In addition, the interaction of Ca^{2+} with ATP, ADP, and P_i is negligible compared with that of Mg^{2+}, because a cation concentration as low as 10 μM is sufficient for full activation of the enzyme. Thus the net effect may be regarded as the ATP hydrolysis when MgATP is added to the SR vesicles in the calorimetric experiment.

Although a systematic study of the variation of ΔH with temperature, pH, $[Mg^{2+}]$, and ionic strength has not been made, the values obtained so far under various conditions are useful for the present analysis and summarized in Table 2. The calorimetric results are generally in good agreement with those obtained by calculation, providing a valuable confirmation of the correctness of the latter values and vice versa, and ΔC_p for the ATP hydrolysis could be calculated for the first time from the calorimetric results. The best estimate is 0.17 ± 0.03 kJ $\cdot$ K^{-1} $\cdot$ mol^{-1}, which also agrees with the value assumed by Alberty (5).

C. *Nature of High-Energy Phosphate Bond*

Since Lipmann (170) proposed the concept of "energy-rich" phosphate bonds, much experimental and theoretical effort has been made to explain why ATP as well as some other phosphate compounds are thermodynamically

[6] In this work, P^1,P^5-di(adenosine-5')pentaphosphate, which is the potent inhibitor of adenylate kinase (169), was used to prevent further breakdown of ADP formed from the hydrolysis of ATP (164).

unstable. In previous formulations the high-energy property of these compounds was thought to be mainly intramolecular in origin. Thus either singly or together intramolecular effects such as opposing resonance, electrostatic repulsion, and electron distribution along the P–O–P backbone might play an important role in the large negative free energy of hydrolysis of high-energy compounds.

George et al. (95), however, pointed out that the importance of the solvent water had been overlooked in these earlier studies. Analyzing the thermodynamics of hydrolysis of different pH forms of pyrophosphate (PP_i), acetyl phosphate, and phosphoenol pyruvate, they suggested that differences in solvation energy of reactants and products made the predominant contributions to the large negative free energies of hydrolysis of these compounds, whereas the intramolecular effects were of secondary importance. This suggestion was later confirmed by the quantum chemical calculation of Haynes et al. (117).

These studies indicate that if the hydrolytic reactions of various high-energy compounds took place in the gas phase (nonaqueous environment), reaction energies would be strongly positive. In contrast, in the aqueous phase the reaction energy would be negative, having values that are in good agreement with available experimental reaction enthalpies. The importance of the solvation (hydration) effect has a most intriguing bearing on the reaction mechanisms of ATPases, because the enzyme proteins would probably provide a catalytic environment for the reaction that is grossly different from those in the bulk aqueous phase. This problem is discussed in sections VIII and IX.

VI. KINETIC MECHANISM OF ATP HYDROLYSIS BY MYOSIN AND ACTOMYOSIN

A. Introduction

The molecular entities responsible for muscle contraction are the proteins myosin and actin, which are arranged separately into the thick filaments and the actin filaments in living muscle. The thick filaments have regularly spaced projections consisting of the globular head of the myosin molecule that contains the sites for ATP hydrolysis and interaction with actin. During muscle contraction these projections of myosin heads form cross bridges with the actin filaments, which attach and detach cyclically. As a result of the interaction the relative sliding force is developed between these filaments. The energy for this physical process is provided by ATP hydrolysis, which is catalyzed by the molecular complex of myosin and actin. This is the actomyosin ATPase activity in vivo, which can be studied in vitro with purified myosin and actin.

B. Contractile Proteins

The myosin molecule is large (M_r 500,000) and consists of a long tail region carrying two globular heads that can be visualized by electron microscopy (76, 172, 215). The tail is insoluble at low ionic strength and is responsible for the aggregation of myosin into thick filaments. Each of the globular heads has an enzymatic site that binds ATP and subsequently hydrolyzes it, and each has a binding site for actin. These two sites are distinct but can interact with each other (see sect. VII*B3*).

The myosin molecule from skeletal muscle consists of two polypeptide chains with molecular weight of 200,000 (heavy chains) and four smaller peptide chains with molecular weights from 16,000 to 27,000 (light chains) (84, 296). The light chains are divided by operational convention into two classes. One class of light chains can be selectively released by treatment with the thiol reagent DTNB. The other class of light chains is dissociated at high pH and therefore called the alkali light chains. Two distinct alkali chains exist, alkali 1 (A_1) and alkali 2 (A_2) with molecular weights of 21,000 and 17,000, respectively. These two chains are the same except for an extra 40 amino acid residues in the NH_2-terminal region of A_1 chain. There are three types of light-chain combinations in myosin molecules: $(DTNB)_2 + (A_1)_2$, $(DTNB)_2 + (A_2)_2$ (131), and $(DTNB)_2 + (A_1) + (A_2)$ (130).

For many biochemical purposes, the heads are separated from the tail by proteolytic digestion (172), and studies can then be made on heads either singly (subfragment 1, SF-1) or in pairs joined by a short part of the tail (heavy meromyosin, HMM). These proteolytic subfragments are soluble at or below physiological ionic strength, unlike parent molecules. Evidence obtained so far indicates that they retain the enzymatic and actin-binding properties almost unchanged compared with those of the intact myosin.[7]

The unique enzymatic property of myosin is its low ATPase activity that is strongly activated by actin (see sect. VI*C3*). The turnover rate of the catalytic cycle is around 0.03 s^{-1} at 20°C under physiological conditions (0.15 M KCl, 5 mM Mg^{2+}, at neutral pH). The situation may be analogous to that in resting muscle in which interaction with actin is prevented.

Actin is a relatively small, globular protein (M_r 43,000). The monomers (G-actin) have a strong tendency to form double-stranded linear polymers (F-actin) at physiological ionic strength. It is this form of actin that exists as the thin filament in muscle and is used for experiments in vitro. The unique property of actin is its ability to activate the ATP hydrolysis by myosin. Under appropriate conditions the actin activation is several 100-

[7] Recently it has been shown that the heavy chain alone contains the catalytic site for ATP hydrolysis and that neither class of light chains is essential for activity (254). From this point on, myosin refers to its head portion unless otherwise specified.

fold, which compares with a factor of ≃1,000 for the relative turnover rates of muscle shortening at maximum velocity and resting muscle (see ref. 81).

Physiological investigation of muscle contraction has largely concentrated on frog muscle, whereas rabbit muscle has been the subject of extensive biochemical studies. It is desirable to correlate physiological and biochemical data obtained with material from the same biological source for better understanding of the mechanism of muscle contraction. Recently some efforts have been made to isolate contractile proteins from frog muscle and characterize their kinetic properties (79–81). Although frog muscle myosin and its subfragment are too labile to perform extensive biochemical studies (79), the data obtained so far indicate that the mechanism of frog myosin ATP hydrolysis is similar to that of rabbit myosin.

C. Mechanism of ATP Hydrolysis by Myosin and Actomyosin

Kinetic studies have revealed that the hydrolysis of ATP in vitro by myosin and actomyosin is complex and consists of a sequence of intermediate reactions, which may be simplified for this discussion as the cycle of reactions shown in Figure 1. For more information, excellent reviews are published almost every year (e.g., refs. 1, 140, 184, 255, 274, 278, 281).

1. Myosin ATPase reaction

The most characteristic feature of the myosin ATP hydrolysis is that, when the reaction is started by mixing an excess of ATP with myosin and

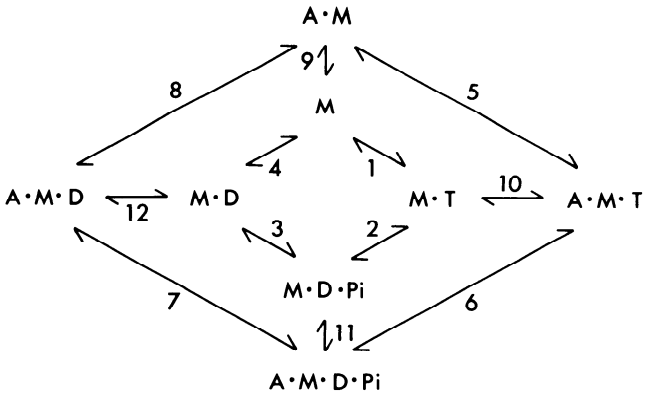

FIG. 1. Kinetic scheme for myosin and actomyosin ATPase reaction. *Inner circle* represents myosin ATPase cycle in absence of actin. *Outer circle* represents ATP hydrolysis by complex of actin and myosin. A, actin monomer in F-actin filament; M, single myosin head; T, ATP; D, ADP. For simplicity, all 2-step processes involving binding and release of ATP, ADP, P_i, and actin are shown as single steps.

then quenching it with acid at intervals to measure the progress of the reaction, one observes a rapid burst of P_i formation. This phenomenon, called the early phosphate burst, proved to be due to the acid denaturation of the protein, releasing P_i from the myosin product complex that undergoes slow dissociation during the normal enzyme catalytic cycle. Thus the myosin ATPase cycle can be divided into two parts, the steps in hydrolysis and the steps in product release. This unique property has facilitated the kinetic analysis.

An important feature shared with a number of enzyme reactions is that the binding of a ligand occurs in at least two steps (for reviews see refs. 110, 143, 144): collision-controlled association followed by an intramolecular change in the protein (isomerization). The collision intermediate is in rapid equilibrium with free forms of the enzyme and the ligand; the equilibrium constant is usually on the order of 10^3–10^4 M^{-1}. The next step, isomerization, usually has a large equilibrium constant, resulting in the tight binding of the ligand. It is in this step that a unique change in protein conformation takes place, depending on the nature of the ligand. Note that in the scheme shown in Figure 1, steps involving interaction of the protein with the ligand are known to occur by such a two-step mechanism (9, 17, 42).

The first step in the reaction between ATP and myosin is the binding (step 1 in Fig. 1). In this reaction the forward rate of the isomerization is very fast (>1,000 s^{-1}; see refs. 44, 145) and the reversal is very slow (<0.01 s^{-1}), so the overall equilibrium constant is on the order of 10^{11} M^{-1} (35, 101). In addition the rate of ATP dissociation from myosin is much slower than the rate of ATP hydrolysis (step 2) and release of products (steps 3 and 4). Thus the binding of ATP to myosin is effectively irreversible.

The next hydrolysis step is characterized by a rather small equilibrium constant that ranges between 1 and 10 depending on conditions (12, 45, 273). This is a remarkable contrast to the situation in free solution, in which the equilibrium strongly favors the hydrolyzed state (see sects. V and VIII*B2*). The forward reaction is moderately rapid; the rate constant is in a range between 20 and 200 s^{-1} (45, 92, 145, 275). Thus the hydrolysis is faster by at least two orders of magnitude than the rate of the overall catalytic cycle.

When the ATP is hydrolyzed on the myosin site, changes in microenvironments of protein side-chain groups take place, as detected by various methods. These include a variety of spectroscopic methods (19, 204, 300), measurements of the deuterium-hydrogen exchange rate (310, 311), and reactivity of protein side-chain groups toward the specific modifying agents (210, 289). Results of recent kinetic studies favor the view that the hydrolysis step, rather than the binding, is responsible for the proton release (41, 43) that occurs through the shift of pK values of the ionizing groups in or near the catalytic site of the protein (274).

The conformational changes in the myosin molecule associated with the hydrolysis are partially reversed when the myosin-product complex slowly dissociates P_i and converts to the myosin-ADP (M · D) complex (step 3). This

reaction is the rate-limiting step in the ATPase cycle at temperatures >10°C (13). Interestingly, however, its apparent rate shows little temperature dependence. On the other hand, the rate of subsequent ADP dissociation (step 4) is strongly dependent on temperature ($Q_{10} > 4$ between 10 and 20°C), and indeed it becomes comparable with that of step 3 at temperatures <5°C. The isomerization that precedes the dissociation is probably the step that is influenced by temperature in such an extraordinary way (see sect. VIII*C1*).

As for the H^+ release, the major contribution is made by step 3, which is accompanied by P_i dissociation, but a fraction of H^+ is liberated in step 2, and the same amount of H^+ is absorbed in step 4. In Table 3 the equilibrium constants for the intermediate steps of the myosin ATPase cycle are listed.

2. Interaction of myosin with ATP analogues

A large variety of ATP analogues has been synthesized during the last two decades (321). The analogues with modified triphosphate moiety have been widely used in studying the mechanism of ATP hydrolysis by myosin and actomyosin (281) and the structural and mechanical properties of glycerinated muscle fibers (102). Relevant to the present analysis are adenyl-5′-yl imidodiphosphate (AMPPNP) and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS).

In the former the nonbridging γ-phosphoryl oxygen atom of the original ATP is replaced by sulfur, so that the kinetics of hydrolysis by myosin is altered. The hydrolysis products are ADP and thiophosphate. Thus the binding of ATPγS to myosin is as fast as that of ATP, but its hydrolysis rate is reduced by some two orders of magnitude and is in fact rate limiting (10, 13, 92, 100). The predominant steady-state intermediate of the myosin-catalyzed hydrolysis is the myosin-substrate complex $M \cdot ATP\gamma S$ in contrast with the myosin-products complex $M \cdot ADP \cdot P_i$ in ATP hydrolysis. However, the subsequent product release takes place much faster than the corresponding step in the ATP hydrolysis. In the calorimetric experiments this analogue was used to study the substrate-binding step, as described in section VII.

The structure of AMPPNP is very similar to that of ATP except that the β,γ-bridging oxygen atom of the latter is replaced by an imino (NH) group (322); hence the analogue is nonhydrolyzable (323).

3. Actomyosin ATPase reaction

On the basis of pre-steady-state kinetic experiments, Lymn and Taylor (175) proposed a simple mechanism of the ATP hydrolysis by actomyosin, which is essentially the pathway of ATP hydrolysis consisting of the following steps from Figure 1. In this mechanism, ATP causes rapid dissociation of

actomyosin (steps 5 and 10), which is followed by ATP hydrolysis on myosin (step 2). The myosin-products complex then reassociates with actin (step 11), and the actin causes rapid release of the products (steps 7 and 8). Because this product release is much faster than the corresponding step with myosin alone, the rate-limiting step of myosin ATP hydrolysis is bypassed in the presence of actin.

Thus this scheme elegantly explains the apparently contradictory classic finding that actomyosin is essentially dissociated at ATP concentrations normally used to measure the ATPase activity, although the rate of hydrolysis is much higher than for myosin alone. In addition a very attractive feature of the model is that myosin and actin undergo dissociation-association reactions for each catalytic cycle of ATP hydrolysis, providing a possible way to correlate the biochemical events (intermediate steps of ATP hydrolysis) with structural events during muscle contraction that involve cyclic dissociation and reassociation of the cross bridges.

Although subsequent studies confirmed that the original Lymn-Taylor scheme fits the kinetic behavior of actomyosin at low actin concentrations, they faced serious difficulties in explaining the steady-state ATPase rate as a function of actin concentration and kinetic behavior of various intermediate states at higher actin concentrations. The scheme has been modified and expanded by kinetic experiments and equilibrium studies have been performed in recent years.

The addition of a step in which ATP is hydrolyzed on actomyosin (step 6 in Fig. 1), i.e., without dissociation of actomyosin, is fundamentally important (258). This nondissociating pathway was, in fact, originally indicated by Tonomura and his collaborators as early as the late 1960s (see ref. 278, p. 191–235). Now there seems to be little doubt that the ATP hydrolysis occurs without dissociation of actomyosin, particularly under conditions with low ionic strength.

Another modification that has long been claimed by Eisenberg and coworkers (1, see also ref. 274) is an inclusion of the refractory state. This state originally referred to the myosin state, which cannot interact with actin, and was introduced to explain the steady-state ATP hydrolysis by actomyosin (75) but now refers to the state before the rate-limiting step, whether myosin is dissociated or associated (1). It is still controversial, however, whether the refractory state is really needed.

It must be admitted that the present state of our understanding of the actomyosin ATP hydrolysis is by no means complete and that rate constants for various steps, particularly transitions between nondissociated states, have not yet been determined unequivocally. However, many attempts have recently been made to determine the equilibrium constants (K_i values) for the steps at which actin binds to the various myosin intermediate states. If these values were known, those for transitions between undissociated actomyosin intermediates could all be estimated by applying detailed balance to the cyclic processes (sect. II*A5*).

Actin binding to nucleotide-free myosin has, of course, been most thoroughly studied under various conditions. Recently, accurate kinetic analysis has also been made possible (188). The second-best-studied step is the actin association to the M · D complex (step 12). The reported values of K_{12} (105, 190) agree well with each other. Thus the dissociation constant of ADP from the A · M · D (step 8) can be calculated. The remaining two actin association steps are hard to analyze precisely. Semiquantitative estimation of K_{10} and K_{11}, however, may be made based on the results obtained by Stein et al. (258). They observed that the actin binding is weakened ~3,000-fold in the presence of ATP or ADP + P_i, where the nucleotide site is probably occupied either by ATP or its hydrolysis products. Thus a reasonable estimation is

$$K_{10} \simeq K_{11} \simeq 10^4\ M^{-1}$$

This estimation is close to the value obtained by a study with a technique of fluorescence-correlation spectroscopy (27). Accordingly, the equilibrium constants for the ATP hydrolysis on actomyosin complex (step 6) and the P_i dissociation (step 7) can be estimated. The equilibrium constants estimated in this way are listed in Table 3.

A most crucial aspect of the actomyosin ATP hydrolysis is that the nucleotide and actin-binding sites on a myosin head are functionally linked

TABLE 3. *Equilibrium constants for intermediate steps of myosin and actomyosin ATPase reactions*

Steps	Reactions		Equilibrium Constants	Refs.
1	ATP binding to M		$5 \times 10^{+11}\ M^{-1}$	35, 101
2	ATP splitting on M		10	12, 45, 281
3	P_i dissociation from M · ADP · P_i		0.1 M	281
4	ADP dissociation from M · ADP		10^{-6} M	154, 281, 288
5	ATP binding to A · M		$5 \times 10^{+8}\ M^{-1}$	
6	ATP splitting on A · M		10	
7	P_i dissociation from A · M · ADP · P_i		10 M	
8	ADP dissociation from A · M · ADP		10^{-5} M	
9	Actin association with	M	$10^{+7}\ M^{-1}$	104, 105, 120, 190
10		M · ATP	$10^{+4}\ M^{-1}$	1, 27, 258
11		M · ADP · P_i	$10^{+4}\ M^{-1}$	1, 27, 258
12		M · ADP	$10^{+6}\ M^{-1}$	105
Completion of cycle	ATP hydrolysis		$5 \times 10^{+5}$ M	238

Keeping overall consistency (i.e., K_{app} of ATP hydrolysis), equilibrium constants were estimated on round numbers from reported values that were obtained under different conditions. Values are referred to conditions of 100 mM KCl, 1–5 mM $MgCl_2$, pH 7–8, and 20–25°C. Equilibrium constants for steps 5–8 were calculated as described in section II*A5*.

so that binding at one site influences the other antagonistically. For example, as described above, the strong interaction between actin and myosin is weakened by about three orders of magnitude in the presence of ATP or ADP + P_i (27, 258). Actin in turn weakens the association of the products with the nucleotide site. Much weaker reduction is seen with ADP alone (105, 189). On the basis of these findings together and those obtained with PP_i or ATP analogues such as AMPPNP and ATPγS, it can be generalized that the affinity of myosin heads for actin is altered cyclically while they pass through a series of intermediate states during the ATP hydrolysis cycle. In addition, actin bound to a distinct site modulates the chemistry of ATP hydrolysis at the nucleotide site and thus facilitates product dissociation. In other words, the myosin intermediates are roughly divided according to their affinity for actin into states that bind actin strongly (M and M · D) and weakly (M · ATP and M · D · P_i). These correspond, respectively, to strongly actin-associated states (A · M · D and A · M) and weakly associated states (A · M · ATP and A · M · D · P_i) (72).

A kinetic difference between weakly and strongly associated states is that the former dissociates from actin much faster than the latter (188). As in the case of the interaction of myosin with nucleotide ligands, the binding of actin to myosin occurs by a two-step mechanism regardless of the nature of the ligand bound to the myosin nucleotide site. It involves a collision-controlled association followed by a protein isomerization (102). Again it is the isomerization step that is affected by the nature of the bound nucleotide and determines whether the association with actin is weak or strong.

VII. CALORIMETRY OF ATP HYDROLYSIS BY MYOSIN AND ACTOMYOSIN

A. Introduction

To evaluate the enthalpy and entropy contributions to the free-energy changes of intermediate steps of the myosin ATP hydrolysis, a few efforts have been made to obtain necessary information from the temperature dependence of the equilibrium constants with the van't Hoff relation. For example, Arata et al. (8) did such an analysis for the ATP hydrolysis by HMM and noted that the formation of a myosin-product complex from myosin (HMM) and ATP was associated with a large increase in entropy. As often suggested, however, kinetic measurements are not generally precise enough for evaluation of the enthalpy changes (see sect. III); hence more quantitative arguments may be difficult. Thus the requirement of calorimetric analysis has become obvious.

In this section, results of calorimetric studies of the myosin ATP hydrolysis performed by my laboratory (153, 154, 158–160) are summarized together with an historical overview and brief description of the method of analysis.

B. Historical Overview

The pioneering calorimetric work on myosin ATPase was reported by Yamada et al. (312). They mixed stoichiometric amounts of HMM with ATP or ADP in an LKB precision calorimeter and followed the subsequent heat production with a time resolution of ~10 s. When ADP was added, a rapid heat production was seen. As ADP binds tightly to HMM, this heat presumably comes largely from their binding reaction (the reverse of reaction 4 in Fig. 1). When ATP was used a similar rapid heat production was seen, followed by a slower exponential heat production. The time constant of this slow phase was the same as for the release of P_i from the myosin-product complex (reaction 3). This seems to show that reaction 3 is strongly exothermic. The rapid heat production comes presumably from reactions 1 and 2 together.

The implication from the results of Yamada et al. (312) that the ADP binding is strongly exothermic was challenged by Goodno and Swenson (99) in a detailed calorimetric study. They reported that the heat of this reaction was small, between −4 and −12 kJ/mol. The question was reexamined by Kodama and Woledge (159) using a calorimetric titration technique (sect. III*C1*). Their result clearly showed that the ADP binding to native myosin is strongly exothermic and that the heat of reaction was definitely greater at 12°C than that at 0°C. However, the observations at 25°C suggest the value does not continue to increase with temperature. They suggested that reliable results were hard to obtain at 25°C because of instability of the protein at this temperature. Measurement of the heat of ADP binding to myosin at 25°C was later made by Banerjee and Morkin (18) with a flow calorimeter that can reduce the exposure time for myosin at high temperatures. The result indicates that the binding heat at 25°C is indeed similar to the value obtained at 12°C by Kodama and Woledge (159). Kodama et al. (158) extended the calorimetric titration study to HMM, SF-1, and myosin filaments and obtained the same results for myosin in these different states. These results have left little doubt that the ADP binding, i.e., the reverse of reaction 4 of the myosin ATPase cycle, is exothermic and hence the forward reaction is endothermic, which is in fact consistent with the classic observation by Martonosi and Malik (193) that the equilibrium constant of the ADP binding to myosin markedly decreases with temperature rise. The effect of temperature on the thermodynamics of the nucleotide binding was recently reexamined by Kodama (154; sect. VIII*C1*).

As for the interaction of myosin with ATP, a Calvet calorimeter was initially used, which gave a time resolution of ~20 s (304). Subsequently a calorimeter with improved time resolution was used (sect. III*B3*; 160). These studies confirmed the exothermicity of reaction 3 as suggested by Yamada et al. (312). On the other hand, Swenson and Ritchie (262) observed a rapid heat absorption and a subsequent slow heat production on addition of ATP to native myosin. The rapid heat absorption at the beginning of the myosin/ATP interaction has not been confirmed by more recent work with a tem-

perature range between 4 and 23°C, which indicates rapid heat production at the beginning (153).

There is another point to be noted for calorimetric studies of myosin and other contractile proteins. It has been known for 10 years that part of the energy produced during contraction of skeletal muscle does not come from the hydrolysis of ATP, the hydrolysis of creatine phosphate, or the reactions that resynthesize these energy sources (47, 132, 166). A hypothesis has been suggested that this chemically "unexplained" energy comes from changes in muscle proteins on activation. The results of calorimetric determination show large heat changes accompanying various changes in the state of the actomyosin system and support the hypothesis (47, 132).

C. Kinetic Analysis of Heat Production During Myosin/ATP Interaction

In the following discussion ΔH_i denotes the reaction heat of the ith step in the simplified kinetic scheme of myosin ATP hydrolysis (Fig. 1).

According to the kinetic mechanism outlined in section VI*C2*, when ATP is mixed with excess myosin, a mixture of M · ATP and M · D · P_i complexes forms instantaneously in terms of the time scale of calorimetry. The heat produced by this rapid phase (h_{fast}) is described by

$$h_{fast} = (-\Delta H_1) + K_2/(K_2 + 1)(-\Delta H_2) \tag{4}$$

Because the dissociation of M · D would be negligible under the conditions where myosin is in excess over ADP, the heat produced during the slower phase (h_{slow}), is given by

$$h_{slow} = 1/(K_2 + 1)(-\Delta H_2) + (-\Delta H_3) \tag{5}$$

The rate of this slow heat production is controlled by the rate constant of this step (k_3). The heat produced by time (t) after mixing myosin with ATP is then described by

$$h_{obs} = h_{fast} + h_{slow}[1 - \exp(-k_3 t)] \tag{6}$$

Because the heat for the hydrolysis of ATP ($-\Delta H_{ATP}$) equals the sum of the heat of four intermediate steps, the total heat produced (h_{total}), is expressed by combining *Equations 4* and *5*

$$\begin{aligned} h_{total} &= h_{fast} + h_{slow} \\ &= (-\Delta H_{ATP}) - (-\Delta H_4) \end{aligned}$$

This equation indicates that ΔH_4 can be determined from h_{total}, because ΔH_{ATP} is evaluated in separate experiments (sect. IV*B2*). Thus, calorimetric

experiments will yield estimation of k_3 and ΔH_4, and provided that the K_2 value is known, *Equations 4* and *5* give estimates of $\Delta H_1 + \Delta H_2$ and ΔH_3. Appropriate values of K_2 have been provided by Taylor (273) and Chock and Eisenberg (45).

Another type of calorimetric experiment that is possible to perform and would provide useful information is to follow the heat production after mixing ATP with M · ADP, which can be generated in the calorimeter by the reaction just described. The reaction sequence is similar but preceded by the dissociation of M · D (step 4), and the overall reaction is simply the hydrolysis of ATP. The time course of heat production is divided into two phases—the rapid phase, equal to $(-\Delta H_4)$ plus h_{fast} given by *Equation 4*, and the slow phase, equal to the h_{slow} of *Equation 5*.

With the calorimeter, the response time of which is ~1 s (sect. III*B3*), separate evaluation of ΔH_1 and ΔH_2 is not possible with ATP itself because the cleavage reaction (step 2) is too fast (see sect. VI*C1*). One way of obtaining an estimate of ΔH_1 is to use an ATP analogue that is split more slowly. If the rate of splitting $k_2 < 0.3\ s^{-1}$, which is the upper limit for the calorimeter to follow, the time course of heat production would be composed of h_{fast} and h_{slow}. The fast phase should correspond to binding and the slow phase to splitting and product release together. ATPγS was used in our work (153, 160) because the rate of splitting of this analogue is ~0.2 s^{-1} (10), which is within the possible time resolution of the instrument, and the estimate of ΔH_1 was thus obtained as described below.

It is, of course, desirable to measure the heat of binding of ATP itself, but this is one of the most challenging problems in the energetics of the myosin ATP hydrolysis as well as calorimetry in general, because one needs to construct a stopped-flow calorimeter with a time resolution within 50 ms and sufficient sensitivity to measure a heat pulse as small as 0.5 mJ.

Finally a correction to the ΔH_i values thus obtained must be made for the heat produced by interaction of the buffer components with proton released or absorbed (see sect. III*C2*). The magnitude of such proton movements has been determined with a pH electrode in separate experiments via the protein solution with a reduced buffer concentration (160). The amount of H^+ released for the ATP binding or splitting, i.e., the rapid phase, is ~0.3 mol/mol of ATPase site, and the same amount is absorbed by ADP dissociation (step 4). The P_i dissociation is accompanied by H^+ release of 1.0/site. These results agree well with those obtained by other workers (274). The necessary corrections to ΔH_i values can then be made accordingly.

D. Calorimetry of Myosin/ATP Interaction

Woledge and I (160) observed that on interaction of ATP with excess SF-1 there was a rapid heat production, followed by a slower heat production, as in the reaction sequence shown in Fig. 2*A*. The slow heat production could

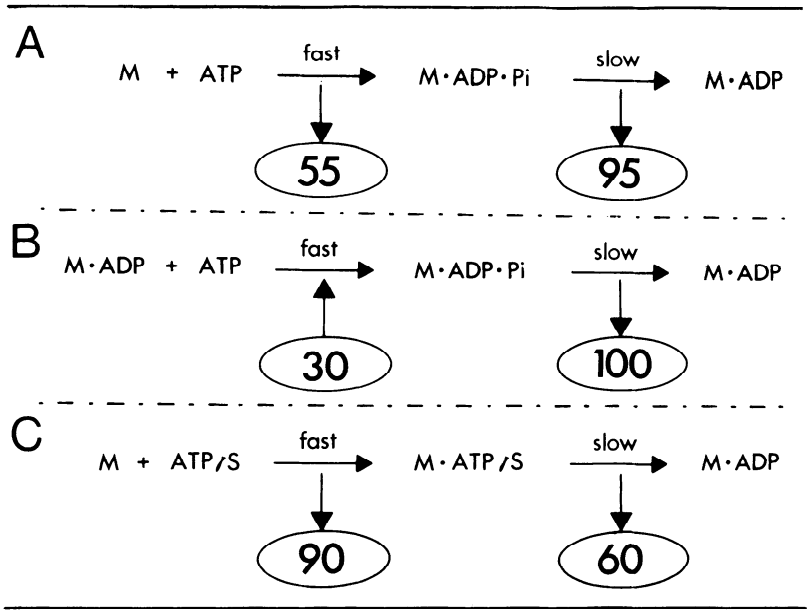

FIG. 2. Schematic diagrams of calorimetric results of interaction of myosin with ATP and 5'-O-(3-thiotriphosphate) ATPγS. Reactions were started in calorimeter as indicated at far left. Myosin (M or M·ADP) was excess over ATP or ATPγS so that end-product was M·ADP in all cases. *Up arrow* represents heat absorption and *down arrows* represent heat production. Figs. in ellipses refer to heat values in kJ/mol of ATP at 23°C and are correct within ±5 kJ/mol. [Based on data of Kodama (153) and Kodama and Woledge (160).]

be fitted by a single exponential, as expected from *Equation 6*. The heat of ADP binding was estimated by subtracting the value of $(-\Delta H_{ATP})$ from the total heat. The value of ΔH_4 thus obtained is +83 kJ/mol at 23°C, which is in excellent agreement with that obtained by a calorimetric titration method (154). The slower heat production accounts for about two-thirds of the total heat observed, and the rate of this phase agrees with that for the dissociation of P_i from the M·D·P_i complex (step 3), as confirmed by the measurement of the UV-absorption change (160) and the H^+ release (153).

When ATP was added to myosin, which should have existed as the M·D complex, a fairly rapid heat absorption was observed at the beginning; it was then followed by a slower heat production (Fig. 2*B*; 153). By quantitative analysis one finds that the total heat equals the heat of ATP hydrolysis, and the time course of the second slower phase is approximately the same as when myosin without bound nucleotide (M) is the starting material. ATP binds to myosin much more strongly than does ADP, so the former will chase the bound ADP from the myosin. Because the ADP dissociation is endothermic and is faster than the P_i dissociation, the heat record obtained on the addition of ATP to M·ADP is interpreted as indicating that the rapid phase of the

myosin ATPase catalytic cycle (steps 4 + 1 + 2) is endothermic and the slower phase (step 3) is strongly exothermic. (Note that it is merely a conventional practice to take the free enzyme as the starting point of the catalytic cycle.)

In contrast to a relatively small heat production in the rapid phase of the ATP/myosin interaction, a large heat burst was observed on the addition of ATPγS, which was followed by a slower heat production, as shown in Figure 2*C* (153, 160). The time constant of the slower phase showed reasonable agreement with that for the rate of ATPγS splitting (10; T. Kodama, D. Gower, and R. C. Woledge, unpublished observation). These results indicate that the binding of ATPγS to myosin is strongly exothermic and that splitting and the subsequent release of the product leading to the formation of M · D complex is also exothermic, but only moderately. The heat of ATPγS binding was evaluated, as in the case of h_{fast} for ATP hydrolysis. On the basis of remarkable similarities in the kinetic behavior of the actomyosin system interacting with ATP and with ATPγS (sect. VI*C2*), it is concluded that the binding heat thus obtained is an estimate of ΔH_1 for ATP.

The calorimetric results calculated with *Equations 4-6* are summarized in Table 4. Two remarkable features are thus revealed by calorimetry. First, large enthalpy changes, compared with the enthalpy change for the overall reaction, accompany intermediate steps of the catalytic cycle, and an exothermic step alternates with an endothermic step. Notice particularly that the actual splitting of ATP on myosin is strongly endothermic, which contrasts with the hydrolysis in free solution that is moderately exothermic. Second, the enthalpy change for each step shows a large temperature dependence, i.e., large heat-capacity changes accompany the intermediate steps. In addition, on close inspection it can be seen that the heat-capacity changes show peculiar temperature dependencies. The implications of these results are discussed in section VIII*C*.

TABLE 4. *Reaction heats for intermediate steps of myosin ATPase reaction*

Steps	Reactions	Temperature, °C		
		4	12	23
1	ATP binding	−65	−92	−89
2	ATP splitting	67	82	51
3	P_i dissociation	−73	−81	−52
4	ADP dissociation	54	72	70
1 + 2 + 3 + 4	ATP hydrolysis	−17	−19	−20

Data for 100 mM KCl, 10 mM $MgCl_2$, pH 8.0. All values (kJ/mol) were corrected for heat from reaction of protons released or absorbed with buffer, assuming that ~0.3 mol H^+ is released by step 2 (41) and the same amount is taken up by step 4, and 1.0 mol H^+ is released concomitant with step 3. Heat values for ATP hydrolysis are from Table 2.

E. Calorimetry of Myosin/Actin Interaction

Relatively little calorimetric work has been done on the reactions of the actomyosin cycle. The fact that actomyosin hydrolyzes ATP much more rapidly than does myosin alone is the major difficulty in such calorimetric studies. In addition, a simple calculation illustrates another difficulty in the experiment.

Suppose a calorimeter, which could measure a heat production of 2 mJ over a period of a few seconds, were used to measure the heat effect of ~40 kJ/mol accompanying an intermediate step (or steps) of the actomyosin cycle. The amount of actin needed to produce a detectable heat would then be calculated to be on the order of 50 nmol, which is equivalent to 2 mg actin in 1 ml of reaction in the calorimeter. As is well known, the actin solution is too viscous to achieve effective mixing suitable for kinetic analysis of heat production. To date, only some preliminary experiments have been performed by Woledge and colleagues (personal communication; see also refs. 47, 304).

The interaction of myosin with actin in the absence of a nucleotide is probably not an essential step in the actomyosin cycle, but the understanding of its thermodynamics is important for various reasons. Because the reaction strongly favors the bound state, the calorimetric titration technique can be used (sect. III*B2*). In an actual calorimetric run, a highly concentrated solution of SF-1 or HMM was added stepwise to the F-actin solution. The reaction is moderately endothermic, ΔH is +30 to +40 kJ for each mole of myosin site binding actin at 12°C. The ΔH is more positive at 0°C. Thus the binding of actin to myosin is driven by a large increase in entropy and accompanied by a substantial decrease in heat capacity. A preliminary estimate of ΔC_p is about −3 kJ/mol. These large changes in entropy and heat capacity are probably due to expulsion of water from the area in which the two proteins approach close to each other.

The reverse of reaction 8 (ADP binding to A · M) also has been studied by the calorimetric titration method. In this experiment, however, it is more difficult to obtain quantitative data as the extent of the reaction, i.e., the formation of the ternary complex A · M · D must be determined in a separate experiment. The result obtained indicates that the heat effect is very small (<10 kJ/mol) in contrast with the large heat production in the corresponding reaction of the myosin cycle (the reverse of step 4).

VIII. ENERGETIC CHARACTERIZATION OF ACTOMYOSIN ATPase SYSTEM

A. Introduction

With all the results for the equilibrium constants (Table 3) and reaction heats (Table 4) of the intermediate steps, it is now possible to depict the thermodynamic profiles of the myosin and actomyosin ATPase reactions.

The free-energy changes for the intermediate steps of these reactions have been considered previously (73, 102, 255, 274, 302).

1. Basic free-energy levels of myosin ATPase intermediates

The basic free-energy levels of the myosin ATPase intermediates are shown in Figure 3 on the basis of the values of the equilibrium constants listed in Table 3, with an assumption that concentrations of ATP, ADP, and P_i are taken as 5, 0.05, and 1 mM, respectively. These values pertain to conditions in muscle in vivo and are generally thought to be effectively clamped constants (47, 48, 90).

As described in section II*A2*, the basic free-energy change is closely related to the kinetics of the transitions between the different intermediate states. According to Simmons and Hill (253), in the basic free-energy diagram a downward change favors flux through the system in the direction of the overall drive (ATP hydrolysis into ADP and P_i), whereas an upward change opposes it. It is the downward basic changes that constitute the stochastic drive at the level of the intermediate in a reaction. Two main features of the myosin ATP hydrolysis are illustrated in the basic free-energy diagram (Fig. 3): *1*) the binding of ATP to myosin constitutes the major drive of the overall reaction; *2*) the $M \cdot ATP$ and $M \cdot ADP \cdot P_i$ states are essentially in equilibrium (isoenergetic).

2. Basic free-energy levels of actomyosin ATPase intermediates

The basic free-energy diagram of actomyosin ATP hydrolysis is shown in Figure 4. In addition to assuming the same values of ATP, ADP, and P_i

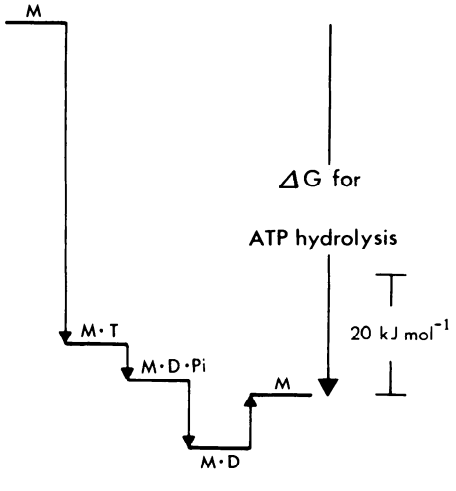

FIG. 3. Basic free-energy diagram for myosin ATP hydrolysis. Basic free-energy changes for transitions between reaction intermediates were calculated as described in sect. II*A2* with equilibrium constants listed in Table 3. Concentrations of ATP, ADP, and P_i were assumed to be 5, 0.05, and 1 mM, respectively.

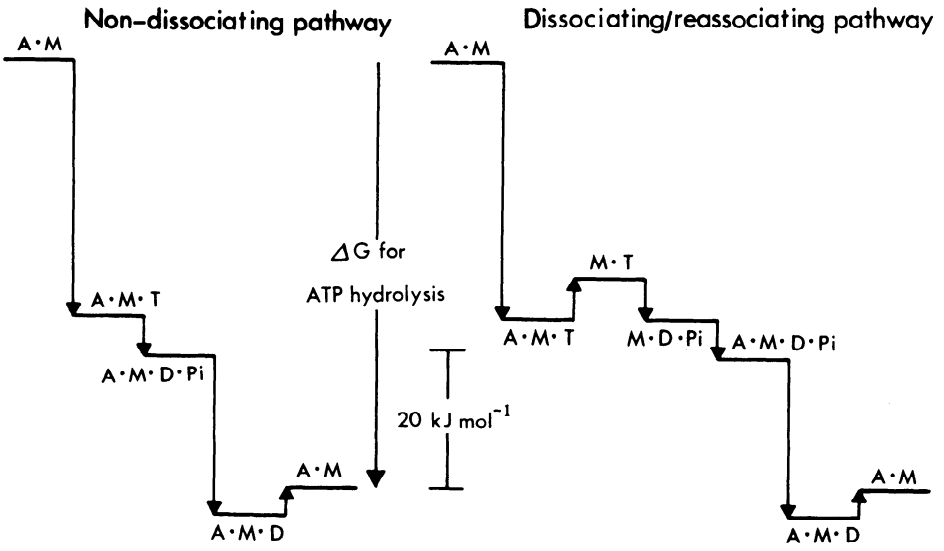

FIG. 4. Basic free-energy diagram for actomyosin ATP hydrolysis. Calculation was made in same way as described in Fig. 3, except actin concentration was assumed to be 1 mM.

concentrations as in the myosin ATPase diagram, it is necessary to assume a value of the actin concentration. The value of 1 mM is used in accordance with Sleep and Smith (255) and Goody and Holmes (102). This value is not much different from the overall average concentration in muscle fibers (~0.2 g actin/g myofibril protein, equivalent to 0.7 mM) (64, 320). As seen in Figure 4, there are two major steps that are associated with large falls in the basic free-energy level, ATP binding to the A · M complex and P_i dissociation from the A · M · D · P_i complex. These two steps correspond to the transition from the strongly to weakly associated states of actomyosin and to the transition in the opposite direction.

An attractive hypothesis that comes out from this fact is that these transitions would be possible candidates for the cross-bridge movement that actually produces force in contracting muscle. This should be considered, however, in taking into account the constraints introduced by the lattice of organized myofibril structure and the observed mechanical behavior of muscle. Discussion of this problem is beyond the scope of this review, and readers are referred to several recent reviews (72, 73, 102, 280).

It is obvious that the large equilibrium constant for the binding of ATP to the actomyosin complex is primarily ascribed to the intrinsic strong binding of ATP to myosin. On the other hand, the thermodynamically stable nature of the M · D · P_i state is responsible for the relatively large energy fall at the P_i dissociation step in the actomyosin ATP hydrolysis. Thus it seems quite appropriate to concentrate on these two steps in the actomyosin ATP hydrolysis. In addition, the nature of the strong association between actin and

myosin and the mechanism of interaction between the actin site and the nucleotide site within the myosin molecule should be considered.

B. Thermodynamic Profiles of Myosin ATPase Hydrolysis

How then are these characteristic features of the myosin ATPase hydrolysis accounted for in thermodynamic terms? This question is the thermodynamic inquiry into the nature of the various forces involved in stabilizing these myosin states and their relative strength. The unitary quantities of thermodynamic parameters for the intermediate steps of the ATPase cycle are listed in Table 5 for this purpose.

1. Nucleotide binding

The fundamental reason that ATP binding to myosin is the major drive of the myosin ATP hydrolysis is the extremely large value of the equilibrium constant (10^{11} M), which is equivalent to ΔG_u of about −80 kJ/mol. This large unitary free-energy change is completely accounted for by a large favorable enthalpy change ($\Delta H_u = -90$ kJ/mol). Because the accompanying ΔS_u is small, the M · ATP is described as a state stabilized by a large decrease of enthalpy (internal energy). A favorable ΔG_u for the binding of ADP to myosin (the reverse of the dissociation in the ATPase cycle) is also dominated by a large negative enthalpy change, but the opposing change of entropy ($\Delta S_u < 0$) is much larger in this case.

As described in section IV*B*, thermodynamics of the binding of ATP or ADP to myosin, like the ligand binding to proteins in general, is conveniently treated in the following four processes (which do not necessarily take place in sequence).

TABLE 5. *Thermodynamic parameters for intermediate steps of myosin ATPase reaction*

Reactions	ΔG_u* kJ mol^{-1}	ΔH_u, kJ mol^{-1}	ΔS_u,* kJ K^{-1} mol^{-1}	$\Delta C_{p,u}$,† kJ · k^{-1} · mol^{-1}
ATP binding	−76	−89	−0.04	−0.4§
ATP splitting	−6	+51	+0.19	−2.8
P_i dissociation	+15	−52	−0.23	+2.6
ADP dissociation	+44	+70	+0.09	+0.4
ATP hydrolysis	−23	−20	+0.01	−0.2‡

Values are expressed in unitary quantities and for 100 mM KCl, 10 mM $MgCl_2$, pH 8.0, and 20–24°C. See Table 1 for definitions. * Calculated from Table 3 as described in section II. † Calculated from Table 4. ‡ Calculated from data of Kodama et al. (155). § Calculated from values for other reactions.

a) Transfer of nucleotide from bulk solvent to myosin site. Accompanying this process,[8] water molecules that have probably been oriented around the Mg^{2+} nucleotide and protein groups constituting the myosin site are released ($\Delta S_u > 0$).

b) Interaction at myosin site. A part of the enthalpy loss for the nucleotide binding could be ascribed to the interaction of the purine ring of adenine with the protein moieties. However, because myosin does not have an appreciable affinity for AMP, it seems that the pyrophosphate structure plays a far more important role in both the binding and the binding-induced conformation change of the protein. In fact the relatively strong binding of PP_i ($\Delta G \simeq -50$ kJ/mol; ref. 105) is also accompanied by a large negative ΔH_u (R. C. Woledge, personal communication).

Recently, Matsumoto and Morita (195) made a spectroscopic study of the mode of binding of Mg^{2+} to myosin. The result indicates that, although strong binding of the cation only occurs in the presence of a nucleotide (e.g., ATP, ADP, or AMPPNP), the free cation is bound to the active site independently of the bound nucleotide. This seems to contrast with the general view that the Mg^{2+}-complexed form of ATP is the true substrate of myosin ATPase (234, 297). If in fact this were the case, one should take into account the energy changes accompanying the dissociation of the Mg^{2+} and ATP from the complex on the enzyme-active site (i.e., an exothermic process in aqueous environment; see ref. 4) and those for the binding of the cation and the Mg^{2+}-free nucleotide to the site. No calorimetric experiment relevant to this point has been made.

c) Burying site with bound nucleotide in interior of protein molecule. Several lines of evidence indicate that the Mg^{2+}-nucleotide complex bound to myosin is buried within the protein and inaccessible to bulk solvent (11, 185). This process is probably a part of a binding-induced conformation change and may involve some dehydration phenomenon ($\Delta S_u > 0$).

d) Binding-induced conformation change. Judging from the fact that ATP binding to myosin dramatically weakens the binding to actin, it is generally believed that a certain conformational change must accompany the formation of the M · ATP complex. However, because it is not easy to trap the M · ATP state for conformational analysis because splitting of bound ATP is too fast, there has been little evidence that this state assumes a unique conformation. On the other hand, the M · ADP state has been rather well studied by various methods, as described in section VI*C3*, and it may be inferred that the overall conformation in this state is more compact than that in the nucleotide-free M state. If a protein molecule takes up a compact conformation, it would be associated with a net increase in weak interactions within the molecule (e.g.,

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[8] In regard to the unitary quantities of the thermodynamic parameters, the thermodynamic contribution from the loss of rotational and translational freedom of the ligand may not be taken into account in the first approximation (ref. 70, p. 367).

hydrogen bonds, van der Waals contacts, charge-charge interactions) that contributes to a negative value of ΔH_u. Such a change would be expected to result in a decrease in freedom of intramolecular motions, and hence $\Delta S_u < 0$, as seen in the nucleotide binding.

2. ATP hydrolysis on myosin

Using the oxygen-exchange technique, Trentham and colleagues (92, 93, 290–293) have shown that ATP hydrolysis at the myosin site is a single step with direct transfer of the terminal phosphorus from ATP to water. Thus the $M \cdot ADP \cdot P_i$ contains chemically distinct ADP and P_i, so the energetics can be concerned only with the on-enzyme equilibrium

$$M \cdot ATP + H_2O \rightleftharpoons M \cdot ADP \cdot P_i$$

where H_2O denotes a water molecule directly involved in the reaction to distinguish it from those in the bulk aqueous phase (see below). As is clearly shown in Figure 3 and Table 5, although the $M \cdot ATP$ and $M \cdot ADP \cdot P_i$ states are essentially isoenergetic, the latter is stabilized by a large entropy gain with a concomitant decrease in heat capacity. By comparison with the values of ΔS_u and ΔC_p accompanying protein unfolding (sect. IV*C1*), one may have some idea how large these thermodynamic changes are for an intermediate step of the enzyme catalytic cycle in which the enzyme-bound substrate and bound products are in a rapid equilibrium. This characteristic feature of the energetics of the ATP hydrolysis step is considered in reference to the following two aspects.

a) ATP hydrolysis in hydrophobic environment. As described in section VIII*B1*, bound nucleotide is buried in the interior of the myosin and inaccessible to the bulk solvent. If the hydrolysis of ATP occurs under such conditions, neither reactants nor products would be hydrated; hence the energetics of the hydrolysis should differ from that in the aqueous environment. In other words, gas-phase energetics probably govern the reaction.

There are several high-energy compounds whose ΔH values for hydrolysis are favorably negative in the aqueous environment but less negative or rather positive in the gas phase (117). These changes in energetics of hydrolysis may be attributed to the differences in hydration energy of reactants and products (95, 117). The point may be illustrated by the hydrolysis reaction of pyrophosphate

$$H_3P_2O_7 + H_2O \rightarrow H_3PO_4 + H_2PO_4^-$$

The calculated value of the energy change for this reaction in the gas phase is −4.2 kJ/mol, whereas the ΔH value obtained by calorimetry in solution is −30.5 kJ/mol (117). Although no such theoretical calculation has been made yet for the hydrolysis of ATP in the nonaqueous environment, because

of the complexity of calculation, there would be an intriguing speculation that a similar change in the energetics would apply to the ATP hydrolysis on the myosin and in the aqueous environment. The endothermic nature of the hydrolysis could then be explained in part by the endothermic hydrolysis at the expense of an entropy increase accompanying the protein conformation change, as described below.

As for the rate enhancement of ATP hydrolysis at the myosin site, it has been suggested on the basis of theoretical calculation that the electrostatic-repulsion force contained in P-O-P bonds may play a substantial role in lowering the activation energy for the hydrolysis in the largely nonaqueous environment (117). Such intramolecular effects are damped by hydration effects and hence are of secondary importance in the aqueous environments. Geeves et al. (93) showed in their oxygen-exchange study that the β-phosphate of ADP formed by the cleavage is highly constrained in the protein. This is probably another consequence of the catalysis in the hydrophobic region: coordination of β-oxygen of ATP to electrophilic sites would increase the lability of the terminal O-P bond, thus facilitating rapid cleavage.

b) Microenvironments of protein side-chain groups. As first suggested by Morita (204) on the basis of her measurement of the ATP-induced UV-spectrum change, subsequently confirmed by measurement of intrinsic protein fluorescence (300), it seems that some tryptophan as well as tyrosine residues are buried in the interior of the protein when the $M \cdot ADP \cdot P_i$ is formed but become more exposed again on the conversion of $M \cdot ADP \cdot P_i$ into $M \cdot ADP$ by P_i release. Thus the microenvironments of these protein side-chain groups are probably most hydrophobic in the $M \cdot ADP \cdot P_i$ states. In agreement with this, Yamada et al. (311) found that the rate of hydrogen-deuterium exchange of the tryptophan residues markedly decreased when the $M \cdot ADP \cdot P_i$ was formed from the reaction of the myosin with ATP but increased again at the step of $M \cdot ADP \cdot P_i$ to $M \cdot ADP + P_i$.

If tryptophan residues are buried in the hydrophobic region within the myosin molecule, there would be a possibility that some water molecules that have surrounded these hydrophobic residues are set free, resulting in $\Delta S > 0$ and $\Delta C_p < 0$. A similar effect could also be expected to occur in the change of the affinity of myosin toward actin. The affinity in the $M \cdot ADP \cdot P_i$ state is as low as in the $M \cdot ATP$ state (258). Because the interaction of these two proteins is strongly hydrophobic as well as electrostatic in nature (see sects. VII*E* and VIII*D1*), some hydrophobic and charged residues are likely to be involved in the interaction. If these residues were transferred from the protein-solvent interface to the interior of the protein accompanying either binding of ATP to the catalytic site or its subsequent hydrolysis, the affinity for actin would decrease and concomitantly a large increase in entropy would be expected.

However, all the changes do not necessarily favor the observed thermodynamic changes. The change of the microenvironment of other protein groups, in a way that is reciprocal to that described above, has also been

suggested (187, 243, 244, 310, 311). Thus one must bear in mind that the magnitude and sign of the thermodynamic parameters reflect a balance between opposing effects that result from the distinct, local conformation changes of the myosin molecule in the accompanying transitions between the M · ATP and M · ADP · P_i states.

C. Transition of Myosin Head Between Macrostates

So far this section has been limited to the data obtained at temperatures around 20°C. However, once the range of temperatures is broadened, another interesting feature of the myosin ATPase can be seen, which is probably directly relevant to the role of myosin in the energy-transduction mechanism.

1. Effect of temperature on proportion of different myosin ATPase intermediates

Bagshaw and Trentham (13) showed that, although the M · ADP · P_i is the predominant steady-state intermediate in the ATPase cycle at around 20°C, the M · ADP state also becomes significant among the intermediate species at temperatures below 10°C. This shows that the rate of the process controlling ADP dissociation from the myosin is markedly temperature dependent (rate = 1.4 s^{-1} at 21°C and 0.07 s^{-1} at 5°C). Subsequently, the temperature-dependent shift of spectral properties of myosin-product complex during the steady-state hydrolysis of ATP was noted by Morita and colleagues (141, 206) and Béchet et al. (19). Thus the spectral properties observed at low temperatures are similar to those induced by ADP. The results could be interpreted in terms of the equilibrium

$$M \cdot ADP \cdot P_i \rightleftharpoons M \cdot ADP + P_i$$

As temperature falls, the equilibrium shifts toward the right-hand side, due to the marked decrease of ADP dissociation rate. Note that this interpretation is apparently consistent with the thermodynamics of this step in the myosin ATP hydrolysis ($\Delta H < 0$ for the reaction from left to right; see sect. VII*D*). Similar temperature-dependent spectral changes with ATP analogues were also observed by these authors (19, 205). The complex formed with AMPPNP at high temperatures was similar to the M · ADP · P_i state in spectral properties but to the M · ADP state at lower temperatures. Morita (205) analyzed the result assuming that in the interaction with myosin this ATP analogue mimics ADP at low temperatures and ADP + P_i at higher temperatures. Thus the interpretation is that temperature change causes an equilibrium shift between the chemical states of myosin, i.e., the distinct states of the myosin defined by the nucleotide bound to the myosin site.

Recently, however, reports have appeared that show that observed phenomena are hard to explain on this basis alone (153, 154, 249–251).

a) Temperature-dependent anomalies of heat capacity changes for intermediate steps of myosin ATP hydrolysis. Table 4 shows the result of a calorimetric study of myosin ATP hydrolysis at different temperatures (153). As can be seen, the reaction heats for the binding of ATP and ADP to myosin become more strongly negative as temperature rises from 4 to 12°C. The apparent heat-capacity change ($\Delta C_{p,app}$) is calculated to be about −3 $kJ \cdot K^{-1} \cdot mol^{-1}$, which agrees with the earlier result for the ADP binding obtained by calorimetric titration (159). The binding heats, however, show little temperature dependence between 12 and 23°C. On the other hand, $\Delta C_{p,app}$ for the endothermic hydrolysis of bound ATP is positive in the lower temperature range (+2 $kJ \cdot K^{-1} \cdot mol^{-1}$) but strongly negative in the higher temperature range (−3 $kJ \cdot K^{-1} \cdot mol^{-1}$). Just the opposite effect is seen for the step of P_i release from the $M \cdot ADP \cdot P_i$ state.

A more detailed thermodynamic study was subsequently made on the ADP and AMPPNP binding at 4-K intervals (154). The results, which are shown in Figures 5 and 6, indicate that large negative values of $\Delta C_{p,app}$ accompany the nucleotide binding to myosin, and the ΔC_p shows a marked temperature dependence like that for the myosin ATPase intermediate steps.

b) Temperature-dependent ^{31}P-NMR spectra of myosin-nucleotide complexes. The ^{31}P-NMR spectroscopy provides useful information concerning the chemical environments of the phosphorous atom and hence its compounds (89, 233). Using this technique, Shriver and Sykes (249) observed that ADP

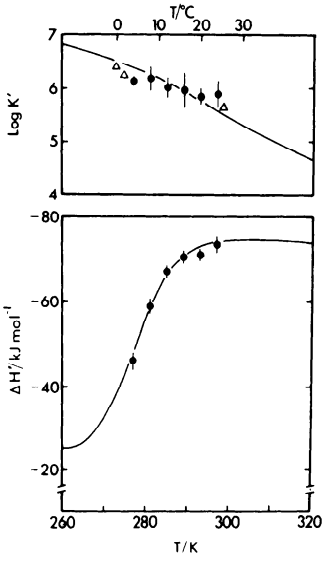


FIG. 5. Variation with temperature of enthalpy change and equilibrium constant for ADP binding to myosin subfragment 1. ●, Data from Kodama (154); △, data from Kodama and Woledge (159). Lines were fitted to points by an iterative least-squares analysis according to *Eqs. 7* and *8* as described in sect. VIII*C2* (see Table 6).

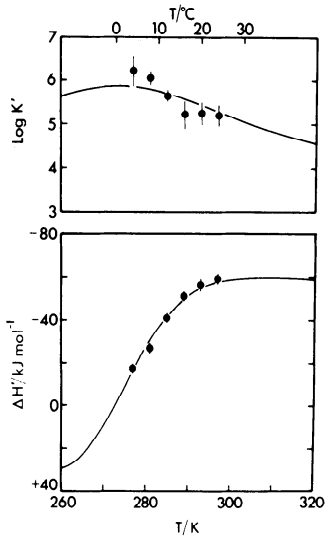


FIG. 6. Variation with temperature of enthalpy change and equilibrium constant for AMPPNP binding to myosin subfragment 1. Lines were fitted as described in Fig. 5. [Based on data from Kodama (154).]

binds to myosin to form one type of M · ADP complex at 25°C, but at 0°C there are two different forms of the M · ADP complex present in nearly equal concentrations. On the contrary, at least two forms of the AMPPNP complex are observed at 25°C, whereas only one form is observed at 4°C. Subsequently, they performed a ^{31}P-NMR study of the ADP binding to myosin over a wide temperature range at smaller temperature intervals and showed that there is a temperature-dependent shift of the equilibrium between the two forms of the M · ADP complex (250). More recently, using ^{19}F-NMR of SF-1 labeled with a fluorine-containing reagent, they also showed that the myosin exists in at least two conformational states even without bound nucleotide (251).

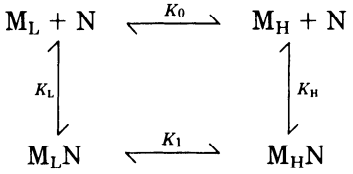
How can all these results be explained? To explain temperature dependence of heat-capacity changes, the two fundamental conformations of myosin were assumed, one of which would be favored by low temperature (M_L), whereas the other (M_H) would exist predominantly above some critical temperature (153, 154). A temperature-induced transition between these states would be expected to modify the thermodynamics of nucleotide binding. Independently, Shriver and Sykes (249, 250) proposed a similar model to explain their NMR results, in which they claimed that the relative populations of M_H and M_L should be determined not only by temperature but also by the nucleotide in the myosin site. It is evident that this hypothesis is essentially the same as the one described for the ligand-induced shift of the equilibrium between two macrostates of proteins (sect. IV*C4*).

The equilibrium between the macrostates can be analyzed based on the data obtained by NMR studies, whereas reaction calorimetry provides the thermodynamic information about the equilibrium between the chemical states with different nucleotides in the active site. Thus these two methods

complement each other in the thermodynamic analysis of the binding of nucleotide and induced conformational change of myosin.

2. *Thermodynamic scheme*

The simplest scheme for the two macrostates of myosin, the equilibrium between which varies with temperature and nucleotide binding, is given by



In this scheme, K_0 and K_1 are equilibrium constants for the isomerization of either the free or the nucleotide-bound myosin from the low-temperature-favored state (M_L and M_LN) to the high-temperature-favored state (M_H and M_HN). The K_L and K_H are the binding constants of N to M_L and M_H, respectively. Likewise, other thermodynamic parameters (ΔH and ΔS) are defined for these processes. These parameters are interrelated with each other, as described in section II. It is assumed that neither isomerization nor nucleotide binding involves a measurable ΔC_p. From the thermodynamic point of view, the two routes taken for the formation of M_HN from M_L

$$M_L + N \rightleftharpoons M_LN \rightleftharpoons M_HN$$

$$M_L \rightleftharpoons M_H + N \rightleftharpoons M_HN$$

are equivalent to each other.

The values of K_0 and K_1 and their temperature dependencies have been determined by NMR studies, hence the corresponding enthalpies (ΔH_0 and ΔH_1) and entropies (ΔS_0 and ΔS_1) have been estimated (250). The apparent binding constant K'

$$K' = K_L(1 + K_1)/(1 + K_0) \tag{7}$$

and apparent heat of binding $\Delta H'$

$$\Delta H' = \Delta H_L + \Delta H_1 K_1/(1 + K_1) - \Delta H_0 K_0/(1 + K_0) \tag{8}$$

can be calculated from calorimetric titration data (154). Note that K_L is related to ΔH_L and ΔS_L by *Equations 2* and *3*. Therefore, taking ΔH_L and ΔS_L as adjustable parameters, *Equations 7* and *8* can be fitted to the observed values of K' and $\Delta H'$ by an iterative least-squares method. The thermodynamic parameters thus obtained are given in Table 6. With these values, the theoretical variations with temperature of K' and $\Delta H'$ for ADP and AMPPNP

TABLE 6. *Thermodynamic parameters for nucleotide binding to myosin in different macrostates*

	Bound Nucleotides	ΔH/kJ mol^{-1}	T_c/K
Transitions between macrostates	None	+103	276
	ADP	+64	271
	AMPPNP	+18	298

	Macrostates	Nucleotides	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}
Nucleotide binding	M_L	ADP	−35	−18
		AMPPNP	+33	+244
	M_H	ADP	−74	−155
		AMPPNP	−52	−69

Left table is conformational transition of myosin from low to high (M_L to M_H) states with different bound nucleotides. T_c, temperature at which standard free-energy change for macrostate transition would be nil (i.e., K_0 or K_1 = 1). Its values were assumed according to Shriver and Sykes (250, 251) to fit *Equations 7* and *8* with data shown in Figures 5 and 6 by an iterative least-squares analysis. Right of table is nucleotide binding to myosin in different macrostates. ΔH_0, ΔH_L, and ΔS_L were adjustable parameters for ADP binding, and ΔH_1, ΔH_L, and ΔS_L were those for AMPPNP binding.

binding were drawn (Figs. 5 and 6). For both parameters of either nucleotide, the curve fitted the actual data well, which clearly shows that the "macrostate-shift" model could account for the observed $\Delta H'$ and K' for nucleotide binding.

The variation with temperature of the population of the two macrostates with and without bound nucleotide was calculated from the best-fit values and is shown in Figure 7. In a lower range of experimentally feasible temperatures, a shift of the myosin macrostate from M_L to M_H takes place on ADP binding, but the magnitude decreases as the proportion of M_H in the absence of nucleotide increases with temperature.

In marked contrast to the ADP binding, AMPPNP binding elicits a large shift from the M_H to the M_L state, which becomes more prominent as temperature rises. This result indicates that the magnitude of the transition from one macrostate to another differs for these nucleotides at a given temperature. For example, at 30°C almost no macrostate shift takes place on ADP binding, because >90% of the myosin exists in the M_H state whether ADP is bound or not. In contrast, about half of the total population of the myosin in the M_H state undergoes a conformational change to the M_L state when AMPPNP binds.

An important conclusion from the analysis described above is that a chemical change at the nucleotide site of myosin elicits a shift of one macrostate to another, and its magnitude depends on the nature of the chemical reaction. Although there has been no direct evidence of whether shifts between the myosin macrostates actually occur during the ATPase cycle, the observed temperature dependencies of ΔC_p for the intermediate steps suggest that it is indeed the case, and the magnitude could be very large in some intermediate steps (153). Thus the macrostate-shift model could show the role of myosin in the transduction of chemical to physical energy in muscle contraction.

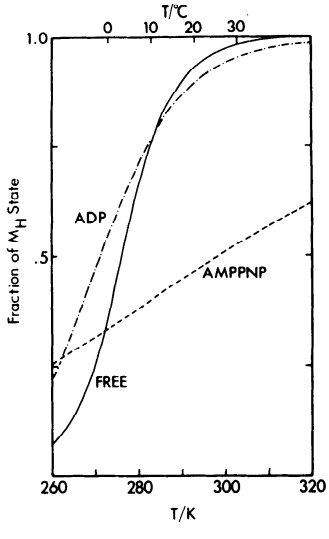


FIG. 7. Population of a myosin macrostate (high-temperature favored, M_H) as a function of temperature and effect of nucleotide binding. It was calculated with values of ΔH and T_c given in Table 6.

3. Energetics

As indicated by *Equation 7* and the relation of $K_0K_H = K_1K_L$, the apparent binding constant K' will always be lower than the intrinsic binding constant of the state with higher affinity for the ligand. This is the manifestation of preferential binding of a nucleotide to one of the myosin macrostates (see sect. IV*C3*), which causes a shift in the distribution of those states. In other words, part of the total intrinsic free energy available for the interaction between myosin and nucleotide is expended to drive the shift of the equilibrium between macrostates.

To illustrate this point, temperature dependencies of K_H, K_L, and K' for AMPPNP binding are shown in Figure 8 on the basis of thermodynamic parameters listed in Table 6. At temperatures above 30°C (which may be more or less physiological for contraction of rabbit muscle), K' is lower than K_L by more than an order of magnitude. Hence the apparent free-energy change for AMPPNP binding is more positive by 6 kJ/mol or more than the intrinsic free-energy change. At the expense of that much energy, about half of the total population of myosin undergoes a shift from the M_H to the M_L state when it binds AMPPNP. This is the coupling of a chemical change to a protein conformational (physical) change that has a certain biochemical consequence, as described below. In the case of ADP binding, which does not cause a large shift in macrostates, the difference between K' and K_H or K_L is not large.

4. Biochemical implications

As discussed by Shriver and Sykes (249), the macrostate-shift model is of particular relevance to its interaction with actin. AMPPNP reduces the

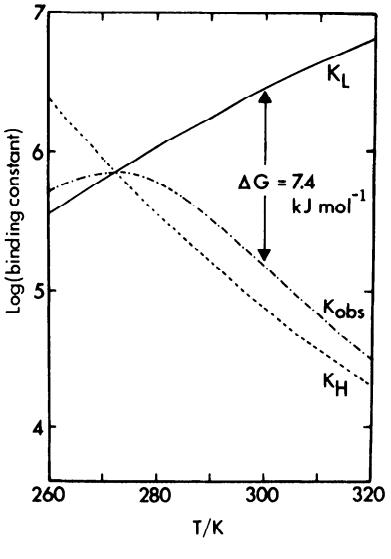


FIG. 8. Variation with temperature of binding constants of AMPPNP to myosin. Observed binding constant (K_{obs}) and intrinsic binding constants to M_H(K_H) and to M_L(K_L) were calculated with values given in Table 6.

affinity of myosin for actin ~10-fold more than ADP does at 25°C, but their actomyosin dissociating effects are not much different at 4°C (105). According to the scheme given here, at ~25°C approximately half of the total myosin population converts from the M_H to the M_L state in the presence of this ATP analogue, in contrast to a few percent shift induced by ADP. On the other hand, there is no marked difference between the macrostate distributions induced by these nucleotides at low temperatures.

Thus the macrostate-shift model is also consistent with the nucleotide-induced modulation of the myosin/actin interaction. The M_L state could be the myosin state with a reduced affinity for actin. In this context, it is interesting to note the report of Marston et al. (189), who observed changes in the tension of muscle fiber in a rigor state in response to the binding of AMPPNP and ADP without appreciable dissociation of cross bridges. AMPPNP is far more effective in this action than ADP. Their interpretation is that there are two states of the actin/myosin interaction that differ in their affinity and cross-bridge orientation. AMPPNP binds to myosin and stabilizes one of these states, resulting in a decrease in the interprotein affinity and a length change without dissociation. On the other hand, ADP preferentially binds to another state but does not induce an appreciable change in the mode of myosin/actin interaction. The exact relationship of these actomyosin states to the myosin macrostates is not certain.

Apart from this direct bearing on the mechanism of muscle contraction, the macrostate-shift model could provide the basis for interpretation of various temperature-dependent phenomena involving myosin. These include the changes in spectral properties of myosin-nucleotide complexes (19, 205) and a biphasic fluorescence response in kinetics of mixing of SF-1 with nucleotides under certain conditions (91, 282). Probably the temperature-induced shift of the UV spectrum of the myosin ATPase steady-state complex (206) could also be explained in terms of the macrostate shift.

In summary, the macrostate-shift model based on the observations by NMR and calorimetry can provide a unified view to account for many of the results from a wide variety of experiments and has important implications for considering the mechanism of coupling between chemical events and a mechanical manifestation in muscle contraction.

D. Energetics of Myosin/Actin Interaction

It has long been known that the interaction between myosin and actin is weakened with increasing ionic strength, decreasing temperature, or in the presence of ATP, PP_i, or ADP (190, 295). Because of improvements in analytical techniques, quantitative data have recently been accumulated, allowing discussion of the thermodynamic aspects of this fundamental interaction between the two principal muscle proteins. In addition, understanding of the substructure of the myosin head has also been enhanced.

Several lines of evidence suggest that it consists of at least three structural domains, and the interaction of these domains appears to be altered when myosin and actin bind together.

1. Entropic nature of interaction

Using the method of time-resolved fluorescence depolarization, Highsmith (120) made a systematic study of the association constant of actin and SF-1 as a function of temperature and ionic strength. He obtained a linear relationship between log K and the reciprocal of temperature over the range between 4 and 25°C, from which the van't Hoff enthalpy was calculated to be +39 kJ/mol (0.15 M KCl, pH 7.0). This value can almost completely account for the apparent difference in the K values determined at different temperatures but at similar ionic strength (e.g., refs. 119, 190). The calorimetric result at 12°C (304) is in good agreement with this van't Hoff enthalpy. Thus it is clear that the actin/myosin interaction is endothermic, and, like many protein-protein association processes (sect. IV*C3*), it is entropy driven (the standard entropy change $\Delta S^0 = 0.3\ \text{kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ at 25°C).

Because there is no evidence that myosin undergoes the delocalized change of conformation, one of the most likely sources of such a large entropy increase seems to be displacement of water from surface areas of two proteins that come in close contact during association. As discussed in the thermodynamic changes for the myosin ATPase reaction, charged and/or hydrophobic side-chain groups are presumably involved in the interaction. The importance of the surface charge can be inferred from the strong ionic-strength dependence of the binding constant. Highsmith (120) also showed that the log K was proportional to the square root of the ionic strength. However, interpretation of this result in terms of the Debye-Hückel theory for the behavior of strong electrolytes in solution is not easy, because the large size and unique shapes of the proteins should be taken into account.

Borejdo (28) recently attempted to map hydrophobic sites on the surface of myosin and actin with a fluorescent hydrophobic probe, *cis*-parinaric acid, which is known to report the surface hydrophobicity of a variety of proteins (149). The result indicates that this probe binds to the region distant from both actin- and nucleotide-binding sites in the myosin molecule, whereas there is little binding to actin. This result, however, does not necessarily preclude the involvement of the hydrophobic interaction in the binding of actin to myosin, because release of water molecules around only several aliphatic chains on binding could be sufficient to account for the observed entropy change. Preliminary results of a calorimetric study indicate that the actin binding to myosin is most likely accompanied by a negative ΔC_p (sect. VII*E*). This suggests that either hydrophobic interaction is important or actin binding induces an equilibrium shift in the macrostate of myosin like that in nucleotide binding to myosin.

2. *Intramolecular changes of myosin on actin binding*

Recently, two totally different approaches have been made to this problem: high-resolution proton NMR (for review see ref. 122) and a proteolytic-digestion method.

Using the former technique, Akasaka et al. (2) found that myosin had a high side-chain mobility within its molecular structure. In a subsequent study, it was shown that the mobile region is almost completely within the myosin head and accounts for ~20% of its structure and that actin quenches these mobilities (123). This effect of actin is not ascribed to a reduced rate of overall molecular rotation on actin binding but rather to the myosin-structure change induced by actin binding (124).

The intact heavy chain of SF-1 (M_r 95,000) is rapidly split by mild treatment with trypsin into three discrete fragments with molecular weights of 27,000, 50,000, and 20,000 (14, 209, 313, 316) that are aligned in this order from the NH_2-terminal of the heavy chain (173). These fragments remain associated in the protein after tryptic digestion but are separable on SDS-gel electrophoresis. A photaffinity-labeling study suggested that the nucleotide-binding site is contained in the 27,000-M_r fragment (263). On the other hand, the 20,000-M_r fragment contained two reactive cysteine residues (SH_1 and SH_2), a chemical modification that drastically alters the enzymatic properties of myosin (14, 286).

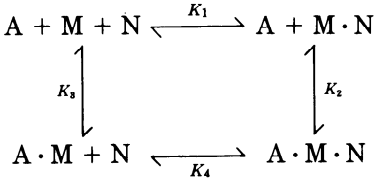
It has been shown that when the tryptic digestion is performed in the presence of actin (actin–SF-1 complex is thus subject to proteolysis), only the 27,000- and 70,000-M_r fragments are produced from the SF-1, indicating that actin induces the structural change within the SF-1 molecule to reduce the susceptibility of the joint connecting the 50,000- and 20,000-M_r peptides to tryptic attack (209, 313). A slight increase in resistance to the tryptic digestion was also noted for the alkali light chains (313). On the other hand, the 27,000- to 50,000-M_r joint seems to become more susceptible to tryptic digestion (313, 314). Mornet et al. (207, 208) argued that the region, the mobility of which is quenched on actin binding as observed by proton NMR, may reside in or near the actin-binding site and that the 50,000- to 20,000-M_r joint is probably part of this mobile structure. Hence actin binding would cause the conformational change of SF-1, resulting in protection of the 50,000- to 20,000-M_r joint from proteolytic attack, and concomitantly the opposite change would occur at the 27,000- to 50,000-M_r joint.

3. *Energetics and mechanism of myosin/actin interaction in presence of nucleotide*

As described in section VI*C*, the nucleotide- and actin-binding sites on myosin are functionally linked in such a way that binding at one site influences the other antagonistically. This is an example of heterotropic interactions

of proteins with interacting sites (306). Because this interaction is fundamentally important to the coupling of chemical and mechanical events in muscle contraction, recent results concerning energetics and the mechanism are summarized here. A different but interesting view of the subject has been presented by Morales and Botts (203).

The interaction between myosin and actin (A) in the presence of a nucleotide or a nucleotide analogue may be described as



If three out of four association constants are determined, the free-energy change of interaction (ΔG_{inter}) can be calculated with the Wyman method (306)

$$\Delta G_{inter} = -RT \ln (K_1/K_4) = -RT \ln (K_3/K_2)$$

This equation describes the strength of interaction between the nucleotide and actin sites. Note that ΔG_{inter} numerically equals the difference between the free-energy changes for binding of either actin or nucleotide to myosin with and without binding of the other.

Table 7 summarizes the values of ΔG_{inter} reported (119, 129) and calculated from the association constants (27, 301) for the interaction of actin and myosin in the presence of ADP, AMPPNP, or PP_i. Partly because of the difficulty of the measurements and the variation in experimental methods and conditions, there are some discrepancies between the ΔG_{inter} values for a given nucleotide. Nonetheless, if the comparison is made for the values obtained at the same or similar ionic strength and temperature, it is clear that when the nucleotide site is occupied with a nucleotide or analogue that has the stronger dissociation power of actomyosin, its interaction with the actin site is stronger. The dissociation power is on the order of AMPPNP $\simeq$ PP_i > ADP.

Only limited information is available for the enthalpy and entropy contributions to the free energy of the site-site interaction. The ADP binding to the A · M complex is almost thermally neutral (see sect. VII*E*). This is consistent with an observation that the binding constant is fairly insensitive to temperature (301). The enthalpy change for the interaction should then be about the same magnitude as for the ADP binding to myosin. Thus the interaction enthalpy is estimated to be −70 kJ/mol. This implies that the interaction between the actin and nucleotide sites is accompanied by a large decrease in entropy. It is of great interest to know how such negative entropic

TABLE 7. *Free-energy changes for interaction between nucleotide and actin sites of myosin head*

Nucleotides	$-\Delta G$/kJ mol^{-1}	Ionic Conditions	Temperature, °C	Refs.
ADP	4.7	A	4	119
	12.2	A	4	105
	7.5	B	22	105
	12.9	C	20	301
	13.1	C	23	129
AMPPNP	12.8	A	4	105
	14.0	B	22	105
	14.0*	A	4	119
	19.4	C	23	129
PP_i	10.8*	A	4	119
	16.2	A	4	105
	15.7	B	22	105

Ionic conditions: A, μ (ionic strength) = 0.17, pH 7.0; B, μ = 0.22, pH 7.0; C, μ = 0.12, pH 8.0. PP_i, pyrophosphate. * Approximate value.

contribution differs for different nucleotides with more favorable free energies of interaction.

The importance of the mobile structure in the mediation of the interaction between the nucleotide and actin sites has been suggested by the proteolytic approach (207, 209, 315), showing that PP_i and ADP only partially impair the protective effect of actin on the tryptic cleavage of the 50,000- to 20,000-M_r joint (a complete loss of the protection was observed in the presence of ATP, but this was attributable to the dissociation of the actomyosin complex). Furthermore, when SF-1 was treated by trypsin in the absence of actin so the 50,000- to 20,000-M_r joint was cleaved, its Mg-ATPase activity was retained, but there was little actin activation. On the other hand, SF-1 digested in the presence of actin showed unaltered actin activation in the ATP hydrolysis. These results suggest that the 50,000- to 20,000-M_r joint plays an important role in activation of myosin ATP hydrolysis by actin.

Chemical modification of the two reactive SH groups in the 20,000-M_r domain affects the properties of myosin ATP hydrolysis, the site for which resides on the 27,000-M_r domain. This fact suggests that there is a close spatial disposition between these domains. In fact, Wells et al. (298, 299) found that when ADP was bound to the nucleotide site (either on addition of ADP or ADP formed from ATP), these SH groups were brought close enough to be cross-linked with bifunctional reagents with cross-link spans of 5–14 Å. The nucleotide was in turn trapped in the active site in a stable manner with a half-life of >7 days at 0°C. Interestingly, however, the trapped nucleotide was rapidly released on the addition of actin. These results suggest

that the chemical event at the catalytic site induces a certain local conformational change in the other domain or domains that are responsible for myosin binding to actins (see ref. 203).

4. Binding of single- and double-headed myosin subfragments to actin

Apart from the functional significance of the double-headed structure of the myosin molecule that has been disregarded in most theories of muscle contraction, consideration of the binding of single-headed (SF-1) and double-headed (HMM) subfragments of myosin to actin is of interest from the energetic viewpoint.

Although it has been known for some time that HMM binds to actin more strongly than does SF-1 (121, 186), the binding process has not been treated quantitatively. The complexity in the analysis of the HMM/actin interaction arises from the "parking problem" (104, 125, 126). This occurs because HMM binds to two actin sites, and as the sites of the F-actin filament become occupied with HMM, the number of pairs of adjacent actin sites available for binding decreases more rapidly than the total number of free-actin sites. However, the problem is not of serious consequence when actin filaments are far from being saturated with HMM.

The binding of HMM to F-actin was studied by the competition method, in which HMM and SF-1 compete for sites on F-actin (104, 106). The data were analyzed with the mathematics for the parking problem worked out by Hill (125). Their results indicate the following:

1. In the absence of nucleotide, HMM binds 100- to 1,000-fold more strongly to F-actin than does SF-1, suggesting that both of the heads of HMM can bind strongly to actin. But the increase in the strength of binding due to the second head is much smaller than what might be expected if each of the two HMM heads bound independently to actin (126, 186).

2. In the presence of ADP or AMPPNP, on the other hand, the binding of HMM to F-actin is not much stronger than that of SF-1. Thus the second-head binding seems to be very weak and in fact does not seem to take place at all in the presence of AMPPNP. A possible physiological implication of nucleotide-weakened, second-head binding of double-headed myosin is discussed by Goody and Holmes (102).

3. Quantitatively, the effect of ionic strength, temperature, and nucleotides on HMM binding is the square of the effect on SF-1 binding. This suggests that the first- and second-head bindings are affected by these agents to the same extent, which implies that the energetics of the intrinsic binding to actin is probably the same for either of the HMM heads as well as for SF-1. Therefore it seems that a smaller contribution of the second head to the free energy for HMM binding to actin would be due to an expenditure of part of the binding energy for protein deformation for two heads to bind actin sites simultaneously under geometrical constraint (cf. ref. 186).

IX. MECHANISM AND ENERGETICS OF ATP HYDROLYSIS BY Ca^{2+}-PUMP ATPase

A. Introduction

The SR serves as the intracellular sink of Ca^{2+}, which has the capacity to release and to reaccumulate the cations. The former is an energetically passive process that is triggered by depolarization-induced redistribution of charges within the muscle fiber membrane and the subsequent similar change in the SR adjacent to the transverse tubule (for reviews see refs. 34, 77, 85, 162, 245). On the other hand, the Ca^{2+} accumulation is the translocation of the cations across the membrane against a chemical potential of Ca^{2+} and hence requires energy input. The molecular entity responsible for this function of the SR is the membrane-bound Ca^{2+}-pump ATPase.

Ever since the pioneering work of Ebashi and Lipmann (63, 65) and Hasselbach and Makinose (115), the mechanism of Ca^{2+} accumulation has been extensively studied with the fragmented SR isolated as a microsomal fraction by differential centrifugation of muscle homogenate. The isolated microsomes consist of small vesicles, 100–200 nm in diameter, and are called the SR vesicles. MacLennan (176) solubilized SR vesicles with deoxycholate and obtained a functional Ca^{2+}-dependent ATPase preparation in a highly purified state. Such purified ATPase preparations have been used for the structural analysis of the ATPase protein. They have also been used in studies of the kinetic mechanism of ATP hydrolysis, the interaction of the ATPase protein with phospholipids, and the reconstitution of SR vesicles with Ca^{2+}-pump activity.

In this section the mechanism of the Ca^{2+} pump is characterized in thermodynamic terms on the basis of experimental data obtained by recent kinetic, equilibrium, and calorimetric studies. For more details of the structure and mechanism of the Ca^{2+}-pump ATPase see several recent reviews (23, 52, 58, 114, 116, 134, 136, 137, 177, 192, 264, 317). The mechanism of free-energy coupling in Ca^{2+} pump and other ion-transport systems has been recently discussed by Tanford (270).

B. Structural Organization of Ca^{2+}-Pump ATPase in Sarcoplasmic Reticulum Membrane

1. Membrane sidedness

When the vesicles are incubated in a medium containing Ca^{2+} and $Mg \cdot ATP$, free Ca^{2+} rapidly disappear from the medium, and the cations are accumulated in the interior of the vesicles (vesicular lumen). This observation indicates that the medium in this in vitro experimental system corresponds to the cytoplasm in the muscle cell (sarcoplasm). In addition, ATP and its

hydrolysis products ADP and P_i react solely from the medium side and do not penetrate the vesicular lumen. Other agents such as ethylenediaminetetraacetate (EDTA) and ethylene glycol-bis(β-aminoethylether)-*N,N'*-tetraacetate acid (EGTA), which are experimentally useful metal-chelating agents, also remain outside the vesicle (294). For convention, in this review the medium and lumen sides are referred to as *out* and *in*, respectively.

2. Asymmetric disposition of Ca^{2+}-pump ATPase in SR membrane

The ATPase protein, which has a molecular weight of 100,000, accounts for 60–70% of total protein in the SR membrane (177). The gross asymmetry has been indicated for its disposition in the SR membrane by biochemical and ultrastructural studies (for reviews see refs. 23, 134, 136, 137, 177, 178, 192, 246, 264). The relatively polar portions of the amphiphilic polypeptide chain of the ATPase protein protrude from the cytoplasmic surface of the membrane. The primary structure of these protein parts has been determined by Allen and colleagues (6, 103; see also refs. 134, 178, 179), whereas the nonpolar portions are largely buried within the membrane, which is probably responsible for a strong interaction with the membrane lipids.

It has been shown with a purified ATPase preparation that ~30 phospholipid molecules are associated with each polypeptide chain of the enzyme (118). These lipids are called annulus or boundary lipids (146, 242). Their interaction with the enzyme protein is stronger than that of other phospholipids, and their removal from the ATPase by detergent treatment results in the irreversible loss of the enzyme activity. Tanford et al. (50, 51, 168), however, showed that these boundary lipids can be replaced by a nonionic detergent, dodecyl octaethylene glycol ether [$C_{12}H_{25}(OCH_2CH_2)_8OH$], without loss of the enzyme activity.

Results of recent studies on the interaction between the ATPase molecules in the SR membrane suggest that the ATPase molecules form a complex (dimer) within the membrane that may be the functional unit for the Ca^{2+} pump (36, 214).

The SR vesicles also contain several minor protein components that are bound much more loosely to the membrane and are easily extracted by a relatively mild treatment with detergents (33, 37, 177). Of these proteins, calsequestrin (M_r 50,000) accounts for 10–20% of the total protein (177), which is generally assumed to be localized on the internal surfaces of SR. Because this protein has a number of Ca^{2+}-binding sites, although its binding constant for the cation is low (~10^3 M^{-1}), it seems likely that calsequestrin molecules confer an increased internal Ca^{2+}-binding capacity on SR. It is known from reconstitution studies that this protein is not essential for Ca^{2+}-pump activity (231).

C. Basic Properties of Ca^{2+} Pump

1. Coupling of Ca^{2+} transport to ATP hydrolysis

The SR vesicles perform rapid Ca^{2+} accumulation when incubated with Mg·ATP together with Ca^{2+}. The maximum capacity of Ca^{2+} accumulation within the vesicle is usually 120–180 nmol Ca^{2+}/mg protein in the presence of excess ATP. The free [Ca^{2+}] inside the fully loaded vesicle is 1.5–2 mM, assuming an average water space of 10 μl/mg protein and the content of calsequestrin in SR and its Ca^{2+} affinity (see above; 217, 218a, 264). Thus most of the intravesicular Ca^{2+} accumulated by the Ca^{2+}-pump activity is present in a bound form.

The rate and the maximum capacity of Ca^{2+} accumulation show a similar dependence on [Ca^{2+}] in the incubation medium: half maximum is observed at pK ~6 and the maximum at pK < 5.5. The Hill coefficient of the saturation curve is ~2. In the absence of ATP, Ca^{2+} also binds, although to a much smaller extent, to the SR vesicle that has high- and low-affinity sites for the cation. The high-affinity sites are attributed to the ATPase protein that has two binding sites per molecule. The binding constant of these sites is ~10^6 M^{-1}. On the other hand, the low-affinity Ca^{2+} sites may be attributed in part to proteins other than ATPase and polar groups of the membrane phospholipids, and it is unlikely that they are directly involved in the Ca^{2+}-pump system.

The ATP hydrolysis rate is very low in the absence of Ca^{2+} (pK > 8) but is significantly stimulated by low [Ca^{2+}] in essentially the same manner as the Ca^{2+} transport. The stoichiometric ratio of Ca^{2+} transported per molecule of ATP hydrolyzed has been measured by a variety of methods under different conditions, and the value is usually two. The same stoichiometric ratio also holds for the translocation of Ca^{2+} coupled to the phosphorylation of enzyme (see sect. IX*D2*).

Thus it is concluded that two Ca^{2+} bind to the high-affinity sites of an ATPase molecule, which then activates the catalysis of ATP hydrolysis, and the completion of the catalytic cycle results in translocation of both cations. [Lower values of the stoichiometric ratio have been reported under certain conditions, in particular at low temperatures, e.g., 0°C (23, 134).]

One of the important properties of the Ca^{2+} pump of SR is that it can be reversed under certain conditions (183). When the Ca^{2+}-preloaded SR vesicles are transferred to the medium deprived of Ca^{2+} with EGTA, but containing ADP and P_i together with Mg^{2+}, there is a rapid Ca^{2+} release to which ATP synthesis is coupled. This is an important demonstration of the reversibility of chemiosmotic energy transduction (58, 114). Note that the stoichiometric ratio of ATP synthesized to released Ca^{2+} is also two, as in the forward mode of the pump function (for more details see sect. IX*D3*).

2. Ion movement accompanying Ca^{2+} transport

For ion movements accompanying the function of the Ca^{2+} pump, the main concern from the viewpoint of energetics is to know whether active Ca^{2+} transport by the pump is electrogenic (net charge displacement) or is accompanied by a displacement of other ions and electrically counterbalanced (for reviews see refs. 116, 201).

Results of recent experiments indicate that some protons are ejected into the medium in exchange for the Ca^{2+} translocation (39, 155, 180, 282a). Note that this proton movement is quite distinct from the net formation of H^+ in the ATP hydrolysis reaction. In addition, when the SR membrane is made permeable to Ca^{2+} by a detergent or a Ca ionophore such as X537A and A23187, proton ejection is not observed, and when the Ca^{2+} gradient formed by the activity of the pump is collapsed by the addition of a Ca ionophore, the released protons are reabsorbed. On the basis of these findings, the proton ejection is attributable to the Ca^{2+} binding to the internal sites of the SR vesicles. Thus the ratio of the H^+:Ca^{2+} exchange depends on the pK values of these sites and intravesicular pH. The value is ~1 at pH 6.0 (39) and ~0.2 at pH 7.0 (155).

As for the movement of other ions, Mg^{2+} is not countertransported during the Ca^{2+} movement (39, 40, 216). Monovalent ions such as K^+, Na^+, and Cl^- are permeable through the SR membrane (40, 116, 201). Thus there is a possibility that the Ca^{2+}-pump activity is electrogenic in itself. However, the buildup of an inside-positive potential across the SR membrane may be largely offset by the inward movement of Cl^- and the outward movement of K^+ under physiological conditions.

D. Kinetic Mechanism of Ca^{2+} Pump

1. Kinetic analysis

A characteristic feature of the ATPase reaction of the Ca^{2+} pump is the rapid formation of a protein-bound acyl phosphate intermediate (phosphoenzyme) followed by its slow decomposition (181, 318, 319). Therefore, like the myosin ATP hydrolysis, the overall reaction is divided into two parts, the steps to the formation of phosphoenzyme and the steps involved in its breakdown and regeneration of the original enzyme state.

Soon after the complete reversal of the Ca^{2+} pump was demonstrated in 1971, it was found that the Ca^{2+}-ATPase could be phosphorylated with P_i in the absence of ATP and Ca^{2+} (with EGTA). The phosphoenzyme formed was subsequently confirmed to be an intermediate in the usual sequence of reaction with ATP (264).

Taking advantage of the acid-stable nature of the acyl phosphate bond of phosphoenzyme on the acid denaturation of the enzyme and participation

TABLE 8. *Kinetic data for partial reaction of ATP hydrolysis by Ca^{2+} pump of sarcoplasmic reticulum*

Hydrolysis Steps	Reactions	Apparent Rate Constants/s^{-1}	Refs.
1 + 2	Formation of E ~ P from E and ATP	80-150	38, 86, 218, 261, 283
5	Dephosphorylation of *E − P	40-60	139, 235
3 + 4 + 5 + 6 + (7)	Decay of *E − P	60	86, 139
Complete cycle	Steady state	8-9‡	139
	ATP hydrolysis	1-2§	218

Values are referring to conditions of KCl 100 mM, $MgCl_2$, 5 mM, pH ~7, and 20-20°C. The term *E − P represents ADP-insensitive phosphoenzyme. † Reaction steps shown in Fig. 9. ‡ In the absence of net Ca^{2+} accumulation. § For intact sarcoplasmic reticulum vesicles.

of Ca^{2+}, a wide variety of techniques are available for transient kinetic studies (38, 86, 136, 137). Several kinetic steps have been identified in the formation and breakdown of phosphoenzyme as shown in Figure 9 (for reviews see refs. 52, 58, 134, 136, 137, 264, 317). Kinetic and equilibrium data for the partial reactions constituting the ATPase cycle are given in Tables 8 and 9, respectively.

The important point here is that during the ATPase cycle, the enzyme protein alternates between two distinct conformational states, referred to as the E and *E states. These two states are distinguished by the property of Ca^{2+}-binding sites and reactivities toward ATP and P_i (58, 134). The Ca^{2+}-binding sites in the E state have high affinity for Ca^{2+} and have an outside

TABLE 9. *Equilibrium constants for intermediate steps of ATP hydrolysis by Ca^{2+}-pump ATPase*

Steps	Equilibrium Constants	Refs.
1 Ca^{2+} binding	$0.5\text{-}6 \times 10^6$ M^{-1}†	20, 58, 61, 133, 138, 284
1 ATP binding	$10^5\text{-}10^6$ M^{-1}	60, 114, 138, 139, 200, 283
2 E ~ P formation	10^{-2} M	224
3 E ~ P ⇌ * E − P	0.5	139, 247
4 Ca^{2+} release	$0.6\text{-}1 \times 10^{-3}$ M†	58, 133
5 *E − P ⇌ * E · P_i	0.6-2.2	59, 147, 148, 191
6 *E · P_i dissociation	$1\text{-}4 \times 10^{-3}$ M	59, 191, 230
7 *E ⇌ E	1/30-1/1,000	191, 271, 284

The terms E ~ P and *E − P represent ADP-insensitive phosphoenzyme. † These values should be squared for interaction of 2 Ca^{2+} with single ATPase molecule.

orientation (facing the external surface of the SR vesicle). In the *E state the Ca^{2+}-binding sites have low affinity and an inside orientation (facing the internal side of the vesicle membrane). In the E state, which is stabilized in the presence of Ca^{2+}, the enzyme reacts with ATP to give rise to one form of phosphoenzyme, E ~ P but not with P_i. On the other hand, Mg^{2+} stabilizes the *E state, in which the enzyme is phosphorylated by P_i in the absence of Ca^{2+} forming the other type of phosphoenzyme, *E − P (see below for distinction between E ~ P and *E − P).

2. Intermediate steps of ATP hydrolysis coupled to Ca^{2+} transport

a) Binding of ATP and Ca^{2+} (step 1). ATP as the complex with Mg^{2+} interacts with the ATPase in the presence of Ca^{2+} to form $Ca_2 \cdot E \cdot ATP$ complex. Because of its inherent complexity (two Ca^{2+} and one Mg·ATP complex participate in the reaction) and subsequent rapid phosphoryl transfer reaction (step 2), kinetic resolution of the process is not yet satisfactory. Evidence for the existence of $Ca_2 \cdot E \cdot ATP$ complex was only recently obtained (224, 248). From equilibrium analysis, however, it has been shown that two Ca^{2+} bind to the enzyme with high affinity in the absence of ATP (see sect. IX*G1*). A reasonable guess of the ATP-binding constant can also be made on the basis of several lines of circumstantial evidence (114, 136, 137). Hence the overall equilibrium constant for the formation of the $Ca_2 \cdot E \cdot ATP$ complex can be estimated (see Table 9).

b) Phosphorylation of the enzyme (step 2). The terminal phosphate of ATP is transferred to a specific aspartyl residue of the enzyme forming a protein-bound acyl phosphate (E ~ P) and releasing ADP into the medium. By analyzing the ATP-ADP exchange reaction, which is a dynamic reversal of steps 1 and 2, it has been shown that free ADP (not complexed with Mg^{2+}) is released (182, 218, 309).

From the viewpoint of Ca^{2+} transport, the most important effect accompanying the phosphorylation is that Ca^{2+} initially bound to the external sites of the enzyme with high affinity is brought into the occluded state in which the cation is inaccessible to EGTA (60, 266-268).

c) Inward orientation and decrease in affinity of Ca^{2+}-binding sites (steps 3 and 4). The rapid formation of the phosphoenzyme is then followed by much slower conformational change of the enzyme (step 3), by which the Ca^{2+}-binding sites become oriented toward the inner surface of SR vesicle, and concurrently the affinity for Ca^{2+} decreases by three orders of magnitude. These changes lead to a release of Ca^{2+} into the vesicular lumen (step 4). The reactivity of the phosphoenzyme with ADP is also altered. As noted above, phosphoenzyme in the E ~ P form transfers its phosphoryl group back to ADP (ADP-sensitive phosphoenzyme), but it becomes unable to react with ADP in step 3 (ADP-insensitive phosphoenzyme, *E − P). It is well known that when the $[Ca^{2+}]$ is increased inside the vesicle by Ca^{2+}-pump

activity, the rate of the pump and ATPase is markedly decreased. This rate limitation is imposed primarily by the "pushing back" of the equilibrium of step 4 by Ca^{2+} and the consequent buildup of the phosphoenzymes.

d) Hydrolysis of phosphoenzyme (steps 5 and 6), outward orientation and increase in affinity of Ca^{2+}-binding sites (step 7). Among all of the partial reactions of the Ca^{2+}-pump ATPase reaction, the best studied is the hydrolysis of phosphoenzyme. It has been investigated by kinetic and equilibrium analysis of its reversal, phosphorylation of the enzyme with P_i as described below. The hydrolysis occurs rapidly and is followed by a relatively slow reversal of the conformational change from the *E to the E state (step 7). The Ca^{2+}-binding site is reoriented to face the outer surface of the vesicle, and the high affinity for the cation is restored. This conversion of the enzyme state is activated by ATP through binding to a low-affinity noncatalytic site (54, 58, 264). Interestingly, the other nucleoside triphosphates such as inosine 5'-triphosphate or other phosphate esters exhibit little stimulatory role, even though they can support the Ca^{2+}-pump activity.

3. Experimental reversal of Ca^{2+} pump

Because the original experiments were performed in the presence of an apparent Ca^{2+} gradient (the Ca^{2+}-loaded vesicles incubated in the Ca^{2+}-deprived medium), the finding of the reversal of the Ca^{2+} pump with Ca^{2+} release coupled to ATP synthesis led to the hypothesis that osmotic energy derived from a transmembrane Ca^{2+} gradient could be utilized for a phosphorylation of ADP. However, subsequent studies (for reviews see refs. 58, 114, 264) have shown that *1)* the Ca^{2+}-ATPase can be phosphorylated with P_i in the absence of a transmembrane Ca^{2+} gradient under certain conditions (see below); and *2)* ATP can be formed by first phosphorylating the enzyme with P_i in the absence of Ca^{2+} (with EGTA) but in the presence of Mg^{2+} and then raising the Ca^{2+} level in the presence of ADP. This indicates that the transmembrane Ca^{2+} gradient is also unnecessary for the phosphoryl transfer from the phosphoenzyme to ADP.

At present the mechanism of the reversal of the Ca^{2+} pump can be explained, if not conclusively, as follows (55, 58). The enzyme is phosphorylated with P_i only in the *E state, where the Ca^{2+}-binding sites are internally oriented with low affinity for the cation. As is evident from the ATPase scheme (Fig. 9), Ca^{2+} shifts the equilibrium between *E and E states toward the latter, as a consequence of formation of the $E \cdot Ca^{2+}$ complex, and hence makes the phosphorylation less favorable. The phosphoenzyme ($*E - P$) thus formed is in equilibrium with $E \sim P$, which has high affinity for Ca^{2+}. The equilibrium constant does not deviate much from unity and is effected by various factors such as the dielectric constant of the medium, temperature, and pH. Subsequently, $E \sim P$ reacts with ADP to form ATP. Thus the enzyme-ATP complex with Ca^{2+} bound to the high-affinity site ($Ca_2 \cdot E \cdot ATP$)

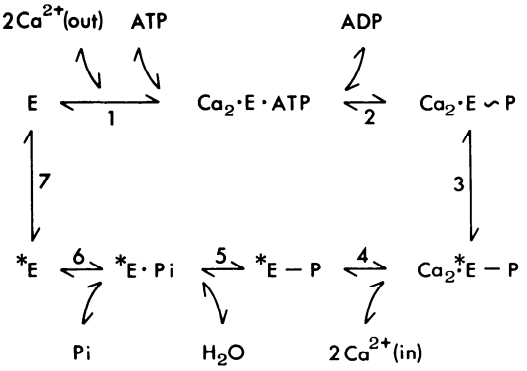


FIG. 9. Reaction scheme for ATP hydolysis coupled to Ca^{2+} transport by Ca^{2+} pump of sarcoplasmic reticulum.

is formed, which dissociates rapidly when Ca^{2+} is released into the low Ca^{2+} medium (i.e., in the presence of EGTA).

From this explanation it is clear why ATP can be synthesized by means of the two-step procedure described above. The ATP synthesis is facilitated by lowering the temperature, for example, in the second step. On the other hand, for the ATP synthesis in the presence of an apparent Ca^{2+} gradient, the large difference of Ca^{2+} concentration on the two sides of the membrane is only needed to meet the differences of affinities of the external and internal Ca^{2+}-binding sites (57, 58). In other words, repeated cycles of ATP synthesis coupled to Ca^{2+} release from the SR vesicle require alternate exposure of the Ca^{2+} sites to an outside medium in which the Ca^{2+} level is sufficiently low to facilitate the phosphorylation of the enzyme with P_i and to an inside environment in which the Ca^{2+} level is sufficiently high to push back the equilibrium from a $Ca_2 \cdot E - P$ to a $Ca_2 \cdot E \sim P$ state (137, 139).

E. Thermodynamic Analysis of Partial Reactions of ATP Hydrolysis of Ca^{2+} Pump

1. Binding of Ca^{2+} to Ca^{2+}-pump ATPase

In agreement with the earlier suggestions based on the Ca^{2+} dependence of the enzyme activity, Ca^{2+} binding occurs in a cooperative manner involving binding to a first site, followed by binding to a second site with a magnitude increase of two orders in the binding constant (138, 284). Kinetic resolution of these two binding processes has been made (61, 135). It has been suggested that the interaction of the enzyme protein with phospholipids and/or the interaction between the enzyme molecules may underlie the apparent cooperative binding of Ca^{2+} (134, 252, 284).

Inesi et al. (138) originally explained the cooperative Ca^{2+} binding in terms of two interacting binding sites. Hill and Inesi (127) subsequently

proposed a more elaborate model that involves interaction of more than two sites, as well as H^+ competition with Ca^{2+} for the binding sites, to explain pH dependence of both the affinity and the cooperativity (287). An alternative explanation for the cooperative character of the Ca^{2+} binding is the shift of the equilibrium between two basic conformational states of the ATPase, E and *E (Fig. 9), which is described below.

Ikemoto (133) and Watanabe et al. (287) found little variation of the apparent binding constant with temperature, whereas Pick and Karlish (223) reported that an increase in the temperature slightly decreased the affinity for Ca^{2+}. Interpretation of these results should be made with caution because, as just described, the Ca^{2+} binding is a cooperative process. In fact, the temperature-induced shift of the equilibrium between the *E and E states may overshadow the actual temperature dependence of the Ca^{2+}-binding constant. In view of these situations it is hardly possible to estimate the van't Hoff enthalpy for Ca^{2+} binding to the Ca^{2+}-pump ATPase.

2. Phosphorylation of Ca^{2+}-ATPase by P_i

This reaction proceeds by way of a ternary complex, $Mg \cdot {}^*E \cdot P_i$, that is formed by random addition of reactants as described by a scheme shown in Figure 10 (161). Equilibrium analysis of this reaction provides thermodynamic information about intermediate steps 5 and 6 of the ATP hydrolysis scheme (Fig. 9).

Using the detergent-solubilized SR vesicles, Kanazawa (147) studied the phosphorylation step ($Mg \cdot {}^*E \cdot P_i \rightleftharpoons Mg \cdot {}^*E - P$) and found that the equilibrium constant is very small (~ 1) and strongly dependent on temperature; the equilibrium favors the phosphoprotein state at higher temperatures. The reaction is thus strongly endothermic, and the phosphorylated enzyme is

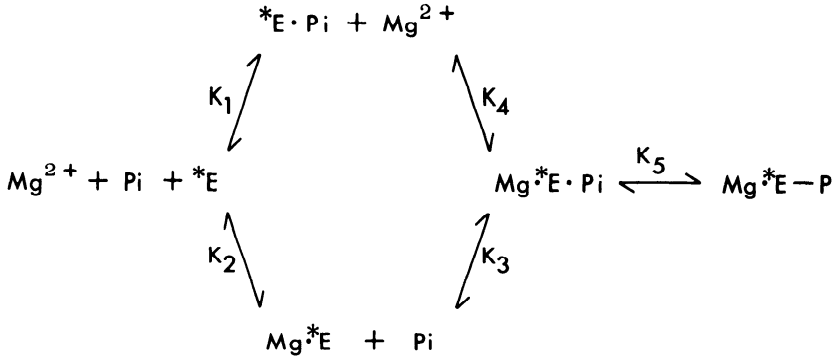


FIG. 10. Reaction scheme for phosphorylation of Ca^{2+}-ATPase by P_i. All equilibrium constants refer to direction of $Mg \cdot {}^*E - P$ formation. Hence $K_1K_4 = K_2K_3$.

stabilized by a large increase in entropy compared with the enzyme-phosphate complex. This important finding has been confirmed with different preparations of the enzyme in several laboratories (20, 148, 191, 194, 235). Kanazawa et al. (148) studied the reaction over a wider range of temperatures (6–38°C) at small intervals and obtained a nonlinear van't Hoff plot. The slope decreases with temperature rise. This result may suggest that the reaction is accompanied by a negative ΔC_p. Considering the difficulty of the experiment, however, further quantitative discussion may not be warranted. The ΔH for the formation of $Mg \cdot {}^*E - P_i$ complex (reaction 6 in Fig. 9) has been estimated by equilibrium analysis of the reactions shown in Figure 10 and their temperature dependencies. Thermodynamic parameters obtained by these studies of the phosphorylation of Ca^{2+}-ATPase by P_i are summarized in Table 10.

Epstein et al. (78) made a calorimetric study of the interaction of Ca^{2+}-ATPase with Mg^{2+} and P_i. This work was an extension of their previous study on the Na^+-K^+-ATPase (165). The result indicated that the binding of either Mg^{2+} or P_i to the enzyme was accompanied by an extraordinarily large negative enthalpy change. The values of ΔH for reactions 1 and 3 in Figure 10 were −96 and −318 kJ/mol, respectively (78). Similar large enthalpy values were obtained for the corresponding reactions of the Na^+-K^+-ATPase (165). On the basis of these results, Racker (232) argued that ion binding induces a conformational change in the enzyme that is prerequisite for phosphorylation by P_i.

Martin and Tanford (191) raised a doubt that the ΔH values obtained by Epstein et al. (78) are not genuine thermodynamic parameters for the processes described by reactions in Figure 10 for several reasons, because the values are not consistent with a modest temperature dependence of the equilibrium constants. For example, an enthalpy change of −318 kJ/mol for Mg^{2+} binding to the enzyme (reaction 3 in Fig. 10) would correspond to a decrease of the equilibrium constant for the reaction by a factor of 74 for a temperature rise of 10 K (at 20°C). This would represent an unusually high temperature dependence for an ion-protein interaction. Martin and Tanford (191) suggested a possibility of a reversible denaturation of the enzyme that might have occurred during lengthy incubation of the unliganded form of the enzyme required for calorimetry. Because transitions of proteins between

TABLE 10. *Thermodynamic parameters for reactions in phosphorylation of Ca^{2+}-pump ATPase by P_i*

Reactions	Steps in Fig. 10	ΔH/kJ mol^{-1}	ΔS/kJ K^{-1} mol^{-1}
${}^*E + P_i \rightarrow {}^*E \cdot P_i$	1 + 4 or 2 + 3	−4.2†	78†
${}^*E \cdot P_i \rightarrow {}^*E - P$	5	+29 to +67‡	10–40

† Data from Martin and Tanford (191). ‡ Reported values vary considerably, depending on experimental conditions (147, 148, 191, 235).

native and denatured states may involve large positive ΔH values (sect. IV), reversal of denaturation by addition of Mg^{2+} or P_i might give anomalously large negative ΔH values for the apparent enthalpy change of ion binding. Note that from a thermodynamic point of view, two routes taken for the formation of the ternary complex $Mg \cdot {}^*E \cdot P_i$, 1 + 4 and 2 + 3, are equivalent, and hence $\Delta H_1 + \Delta H_4$ should equal $\Delta H_2 + \Delta H_3$. However, the data presented by Epstein et al. (78) indicate clearly that this was not the case.

3. *Transition between two fundamental conformational states of Ca^{2+}-ATPase*

Apart from the reactivities toward ATP and P_i and the sidedness and affinity of the Ca^{2+}-binding sites, there is now strong evidence that, whether phosphorylated or not, the ATPase exists in two distinct conformational states.

a) Thermodynamics of conformational transition of unphosphorylated enzyme (step 7 in Fig. 9). There is as yet no way to make a direct approach to this transition in isolation from other steps of the ATPase cycle. Arguments therefore are usually made on the basis of analysis of Ca^{2+} binding and accompanying changes (61, 223, 252, 284) or on studies of Mg^{2+} binding (171, 271) and vanadate binding (223).

An important point suggested from these studies is that the equilibrium between the E and *E states of unphosphorylated enzyme consistently explains the kinetic and equilibrium data of apparent cooperative Ca^{2+} binding. Thus if there is more *E than E in the absence of Ca^{2+}, the first Ca^{2+} binding would involve a conversion of *E to E and hence have a smaller binding constant. The second Ca^{2+} binding would occur on the enzyme already in the E state, and the observed binding constant would therefore be much larger than the first one. In this case, even if the two binding sites have an identical intrinsic affinity for Ca^{2+}, the observed cooperativity of Ca^{2+} binding can be explained by assuming a small value of the equilibrium constant for the transition from the *E to E state, i.e., $K_7 \simeq 10^{-3}$ (271). A similar small value of K_7 has also been inferred from the analysis of Mg^{2+} binding (171). On the other hand, a much larger value ($K_7 \simeq 10^{-2}$) would be acceptable provided that the ratio of the Ca^{2+}-binding constants between the E and *E states is large, say larger than 3×10^3. Thus the *E-state conformation is more stable than E-state conformation by 10–17 kJ/mol.

In an earlier study, Masuda and de Meis (194) suggested that the transition from the *E to the E state is exothermic. Pick and Karhlish (223) estimated an accompanying van't Hoff enthalpy to be −85 kJ/mol. However, their study was performed with a fluorescein-labeled enzyme in which Ca^{2+}-ATPase activity and phosphorylation by P_i are severely impaired. Therefore the observed value may not be the genuine thermodynamic parameter for the native enzyme.

b) Conformational transition of phosphorylated enzyme (step 3 in Fig. 9). The fact that the equilibrium constant of this step is not far from unity was first suggested by the presence of two populations of phosphoenzyme during the steady-state ATP hydrolysis by Ca^{2+}-ATPase (247). Inesi et al. (139) made a kinetic estimation of the ratio of the two phosphoenzyme species and obtained an approximate value of 0.5, which indicates the isoenergetic nature of the two forms of phosphoenzyme. De Meis and Inesi (55) examined the conditions that favor the conversion of phosphoenzyme formed by phosphorylation with P_i to the ADP-sensitive form. They found that when the enzyme was first phosphorylated with P_i at pH 5-6 at 30°C and then transferred to an alkaline medium of pH 8 at 0°C, a large fraction of the phosphoenzyme was converted to the ADP-sensitive form, as judged by the synthesis of ATP. This result may indicate that the transition from the $*E - P$ to the $E \sim P$ state is exothermic. The method is not suitable, however, for estimation of the van't Hoff enthalpy of this transition.

4. Calorimetric studies of ATP hydrolysis by SR vesicles

During the steady state of ATP hydrolysis by SR vesicles in the presence of Ca^{2+}, nearly all the Ca^{2+}-ATPase proteins remain phosphorylated, because phosphorylation is much faster than hydrolysis. Hence it seems that if a calorimetric measurement is made of heat production during the ATP hydrolysis, the total heat observed should be composed of two components, as given by

$$h_{obs} = \xi_{ATP}(-\Delta H_{ATP}) + \xi_{EP}(\Delta H_{EP}) \qquad (9)$$

where ξ_{ATP} is the extent of ATP hydrolysis, and ξ_{EP} is the level of phosphoenzyme in the reaction mixture, whereas ΔH_{ATP} and ΔH_{EP} are the heats of ATP hydrolysis coupled to Ca^{2+} transport and of the phosphoenzyme formation, respectively. Thus, ξ_{ATP} $(-\Delta H_{ATP})$ is due to ATP hydrolysis, and $\xi_{EP}(-\Delta H_{EP})$ comes from the formation of phosphorylated enzyme and accompanying changes such as occlusion of Ca^{2+}. The measurement has to be made at low temperatures (<12°C) to slow down the overall rate of ATP hydrolysis and also in the early phase in which the heat contribution from the phosphorylation would be significant to the total heat production.

Kodama et al. (112, 156) performed such a calorimetric study. Using the same microcalorimeter as for myosin ATP hydrolysis (sect. III*B3*), they observed that there was a rapid heat absorption at the start of the reaction, followed by a slower heat production. They also found that during the first few turnovers of the ATPase cycle, the observed heat was much smaller than the expected heat from the amount of ATP hydrolyzed. This result implies that the formation of phosphoenzyme by the reaction of the enzyme with ATP is endothermic ($\Delta H_{EP} > 0$), because Kodama et al. (155) earlier showed that the ATP-driven Ca^{2+} transport inclusive of Ca^{2+} binding to internal

sites is associated with little heat change after correction for the heat due to the ATP hydrolysis. The value of ΔH_{EP} can be determined from these data with *Equation 9*, and the heat for the phosphoenzyme breakdown can then be calculated as the difference between heats for ATP hydrolysis and for the phosphoenzyme formation.

In this earlier work, however, no correction was made for the observed heat from the interaction of buffer used with protons released on ATP hydrolysis coupled to Ca^{2+} uptake (see sect. IX*C2*). This correction can be made either by measuring H^+ production accompanying the formation and breakdown of phosphoenzyme or by determining the heat values with other buffers having different heat of protonation values, as described in section III*C2*. The latter approach has been adopted in the recent work with MOPS (ΔH_{buffer} = −21.0 kJ/mol) and BTP (ΔH_{buffer} = −43.4 kJ/mol) (157). The results indicate that the heat of phosphoenzyme formation is essentially the same in these two buffers, suggesting that the decomposition of E ~ P is mostly responsible for the liberation of protons accompanying the ATP hydrolysis. The reaction heats for formation and decomposition of E ~ P corrected for heat from the interaction of buffer with these protons are given in Table 11.

F. Thermodynamic Characterization of Ca^{2+}-Pump ATPase System

1. Thermodynamic efficiency of Ca^{2+} pump

The coupling of a transport process to another spontaneous process is a plausible definition of active transport. The spontaneous process, which is

TABLE 11. *Possible sources of large enthalpy changes in Ca^{2+}-pump ATPase reaction*

Reaction Phases	Sources	ΔH/kJ mol^{-1}
Fast, endothermic,†	ATP binding	?
ΔH = +55 kJ mol^{-1}	Splitting of ATP bond	?
	Formation of acylphosphate	?
	ADP release	?
	ADP + Mg^{2+} ⇌ MgADP	+15‡
	Ca^{2+} occlusion	?
Slow, exothermic,†	E ~ P to *E − P transition	Endothermic
ΔH = −70 kJ mol^{-1}	Ca^{2+} release	?
	*E − P → *E + Pi	−24§
	*E to E transition	Exothermic
	Ca^{2+} binding	?

?, Value unknown. † See sect. IX*E4*. ‡ Calculated from values used by Alberty (4). § Calculated from data of Martin and Tanford (191).

often ATP hydrolysis, is the drive for the transport. According to this definition, the total free-energy change for the Ca^{2+} transport into SR by the Ca^{2+} pump (ΔG_{total}) is

$$\Delta G_{total} = \Delta G_{ATP} + n\Delta G_{Ca}$$

where ΔG_{ATP} is the free-energy change accompanying the hydrolysis of 1 mol of ATP, and ΔG_{Ca} is the minimum work required to pump 1 mol of free Ca^{2+} against the concentration gradient, assuming the absence of an electrical potential (see sect. IX*C2*); n is the number of Ca^{2+} taken up per mol of ATP hydrolyzed and is assumed to be 2. In living muscle cells, ΔG_{ATP} is constant and equals −61 kJ/mol at 25°C (sect. VIII*A1*). On the other hand, for the transport process

$$\Delta G_{Ca} = RT \ln ([Ca^{2+}]_{out}/[Ca^{2+}]_{in})$$

where the $[Ca^{2+}]$ refers to the free-cation concentrations in both sides of the SR membrane. With these equations, the efficiency of free-energy coupling can be estimated if a Ca^{2+} gradient ($[Ca^{2+}]_{in}$-$[Ca^{2+}]_{out}$ ratio) maintained by the Ca^{2+} pump is known. It must be recognized that the observed gradient is not a true equilibrium ratio but rather a ratio brought about by the Ca^{2+} pump and passive fluxes in physiological steady states. It is also possible to predict the maximum or thermodynamically possible limit of Ca^{2+} gradient that the pump can achieve.

The physiological level of Ca^{2+} loading of the SR is estimated to be one-fourth to one-third of its maximum filling capacity (77). If it is assumed that calsequestrin is solely responsible for Ca^{2+} binding and buffering inside the SR (see sect. IX*B2*), $[Ca^{2+}]_{in}$ is calculated to be 0.3–0.5 mM. It is estimated that the free $[Ca^{2+}]$ in myoplasm is $\sim 10^{-7}$ M or lower in resting muscle but increases to $\sim 10^{-5}$ M when muscle is stimulated (26, 77). In resting muscle therefore the Ca^{2+} pump should achieve and maintain a Ca^{2+} gradient of larger than 3×10^3, which is much lower than the theoretically possible limit of Ca^{2+} gradient ($\simeq 2 \times 10^4$). In other words, the efficiency of energy coupling in the Ca^{2+} transport by SR is ~70%. This is probably because as $[Ca^{2+}]_{in}$ increases, passive Ca^{2+} leak through the SR membrane becomes significant with concomitant slowdown of the Ca^{2+}-pump activity (see sect. IX*D2*). Note that at $[Ca^{2+}]_{out}$ below 10^{-7} M, the initial rate (in the absence of Ca^{2+} accumulation) of Ca^{2+} uptake and ATP hydrolysis is markedly decreased (217, 294). [It has been mentioned in other reviews that the coupling efficiency of the Ca^{2+} pump is much higher, and the calculated Ca^{2+} gradient is close to the theoretical limit (116, 270). However, the values of $[Ca^{2+}]_{in}$ and $[Ca^{2+}]_{out}$ used in the calculation are different from those in my analysis.]

On the other hand, only 20% of the free energy available from ATP hydrolysis is converted into useful work to take up Ca^{2+} into the SR at $[Ca^{2+}]_{out}$ of 10^{-5} M, and the remainder is used for rate acceleration and dis-

sipated as heat. In fact, the SR vesicle shows the maximum rate of Ca^{2+} uptake and ATP hydrolysis at pK 5 (217, 294).

With the data for the maximum Ca^{2+}-filling capacity of SR vesicles as a function of $[Ca^{2+}]_{out}$ (217, 218a, 294), the Ca^{2+} gradient produced and maintained in vitro can be calculated. The result indicates that the gradient similar to that for conditions in resting muscle are produced and maintained by isolated vesicles.

2. *Basic free-energy levels of Ca^{2+}-pump ATPase intermediates*

The basic free-energy diagram of the Ca^{2+}-pump ATPase intermediates is shown in Figure 11, which is derived from the values of equilibrium constants listed in Table 9.

Three important features of the Ca^{2+}-pump reaction are clearly illustrated in this diagram: *1*) there are two major steps that are associated with large falls of the basic free-energy level, the formation of $Ca_2 \cdot E \cdot ATP$ complex, and the subsequent phosphorylation of the enzyme; *2*) different forms of phosphoenzymes are essentially an isoenergetic transition between which translocation of Ca^{2+} and hydrolysis of phosphoenzyme takes place; and *3*) the reversal of the conformational change, $^*E \rightharpoonup E$, is the major energetically unfavorable process of the Ca^{2+}-pump cycle.

The fundamental reason that the formation of $Ca_2 \cdot E \cdot ATP$ complex is

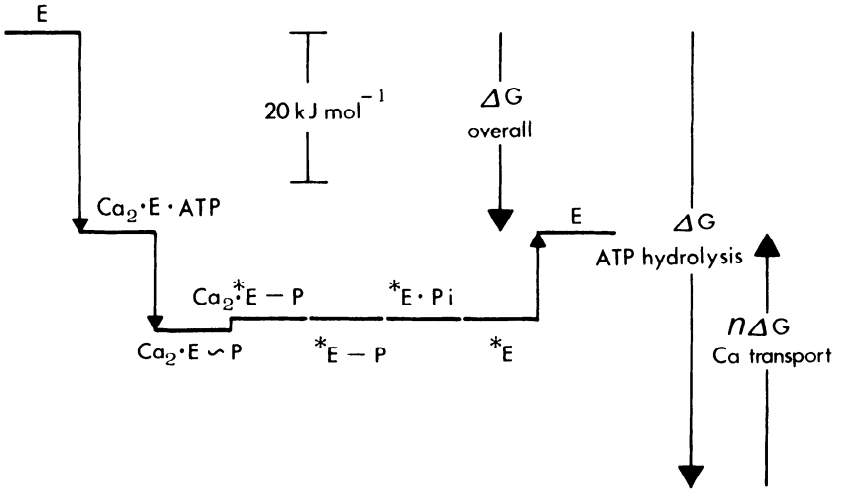


FIG. 11. Basic free-energy diagram for Ca^{2+}-pump ATPase reaction of sarcoplasmic reticulum (SR). Basic free-energy changes for transitions between reaction intermediates were calculated with equilibrium constants listed in Table 9 with $[Ca^{2+}]$ of 10^{-6} and 10^{-3} M for outside and inside of SR vesicles, respectively.

the major drive of the Ca^{2+}-pump reaction is the large equilibrium constants for Ca^{2+} and ATP binding to the enzyme whatever the actual binding sequence in the catalytic cycle. It is understandable that the high affinity for Ca^{2+} of the pump protein facing the external surface of the SR membrane should be prerequisite for binding the cation, particularly when the $[Ca^{2+}]_{out}$ is $<10^{-6}$ M. The high affinity for ATP seems to be essential for the pump to operate when the external Ca^{2+} is lowered further to a level $<10^{-7}$ M, such as in the resting muscle where the equilibrium of the Ca^{2+} binding is apparently not favored.

A substantial fall of the basic free-energy level in the subsequent phosphoryl transfer reaction is mainly due to the dissociation of ADP into the medium that usually (both in the physiological situation and in the ordinary assay system of the Ca^{2+}-pump activity) contains a low concentration of the nucleotide. Indeed, the equilibrium constant for the actual cleavage of the ATP terminal phosphate coupled to phosphorylation of the aspartyl residue of the enzyme is small (Table 9). Thus it is evident that if the ADP concentration in the medium were increased for some reason, the fall in the free-energy level accompanying the phosphorylation would be decreased, and the function of the Ca^{2+} pump would be affected considerably. In fact, Dawson et al. (49) showed in their ^{31}P-NMR study of fatiguing muscle of the frog that the decrease in mechanical relaxation rate is linearly related to an increase in ADP concentration.

Because free ADP is dissociated at step 2 (see sect. IX*D2*), it is desirable to see whether the calculation of the free-energy change of this step is affected by taking this fact into account. Usually, as in this review, concentrations of ATP, ADP, and P_i represent all ionized (free) and Mg^{2+}-complexed species of each component (see sect. III*B*). With the values of the acid-dissociation constants and stability constants of free and Mg^{2+}-complexed species of ADP (4), free-ADP concentration can be calculated, which turns out to correspond to ~65% of the total concentration in the presence of 1 mM Mg^{2+} at neutral pH. Thus this figure does not seriously affect the calculation of the free-energy change for step 2. On the other hand, the contribution of interaction of free ADP with Mg^{2+} to the heat change will not be negligible (see sect. IX*F3*).

The conformational change from *E to E does not proceed spontaneously. Thus energy input is required for the pump to enter into the next cycle. This energy deficit is compensated for by expenditure of a part of the large favorable free-energy change accompanying the Ca^{2+} and ATP binding followed by phosphorylation. The rate acceleration is another matter, however, and ATP may stimulate the transformation of *E into the E state through binding to a low-affinity, noncatalytic site, as described in section IX*D2*. [Boyer et al. (31, 199) recently proposed the regulatory role of ATP in Ca^{2+} pump and other ion-transporting ATPases.]

3. Nature of forces stabilizing Ca^{2+}-pump intermediates

An attempt is made here to assess which step or steps may contribute to the observed enthalpy changes for the rapid formation of phosphoenzyme and its subsequent slow decay in the Ca^{2+}-pump reaction (Table 11). The calorimetric results indicate that the phosphoenzyme formation is endothermic and hence driven by a large increase in entropy, whereas the slow phosphoenzyme decay is strongly exothermic and thereby makes the overall Ca^{2+}-pump reaction (ATP hydrolysis) exothermic (see sect. IX*E4*). In addition, possible sources for large entropy changes during the Ca^{2+}-pump cycle are discussed.

a) Phosphoenzyme formation. Of reactions involved in the phosphoenzyme formation, association of free ADP (released on E ~ P formation) with Mg^{2+} in the medium is the only reaction for which the enthalpy change can be estimated. It is evident, however, that its magnitude is too small to account for the overall endothermicity of the phosphoenzyme formation. Judging from calorimetric results of binding of nucleotides to various enzyme proteins including myosin, it is likely that the Mg^{2+}-ATP binding is itself exothermic.

The enthalpy change for the dissociation of ADP uncomplexed with Mg^{2+} on E ~ P formation is also unknown. According to a recent study of ADP binding to myosin subfragment 1 under various conditions (288), the binding constants in the presence and absence of Mg^{2+} show similar temperature dependencies, suggesting that the dissociation of ADP uncomplexed with Mg^{2+} from the myosin site may be endothermic. Although a simple extrapolation of this result to the Ca^{2+}-pump reaction is not warranted, it is intriguing to speculate that ATP binding and ADP dissociation thermally cancel each other (at least partially). If this were the case, the remaining three reactions (cleavage of the ATP-terminal phosphate, formation of acyl phosphate bond and the occlusion of Ca^{2+} bound to the high-affinity sites) together would be strongly endothermic. Although the nature of the occlusion of Ca^{2+} is not yet known, it can be regarded as a manifestation of a certain conformational change within the Ca^{2+}-ATPase molecule accompanying the E ~ P formation. Thus there is a close analogy with the ATP-cleavage step of the myosin ATP hydrolysis, which is endothermic and accompanied by a certain local conformation change, as described in section VII.

b) Phosphoenzyme decomposition. The overall van't Hoff enthalpy for the process of acyl-phosphate hydrolysis and dissociation of the ternary complex, $*E \cdot Mg \cdot P_i$ (191), can explain about half of the observed enthalpy change of this slow phase of the Ca^{2+}-pump cycle. In addition, the major conformational change, E ~ P → *E − P, seems to be endothermic (139). Thus other exothermic steps should be looked for.

As described in section IX*E3*, the reversal of the conformational state *E → E is probably exothermic. At present even the sign of the reaction heat for Ca^{2+} binding to the high-affinity sites of the Ca^{2+} pump cannot be

speculated. From the enthalpic point of view, there are two classes of Ca^{2+}-binding proteins. Troponin C (226, 307, 308) and parvalbumin (202) show exothermic Ca^{2+} binding. On the other hand, recently, Tanokura and Yamada (272) have shown by calorimetric titration that Ca^{2+} binding to calmodulin is endothermic and driven by a large increase in entropy. An important point is that the binding-site structures are very similar for these three proteins. Thus the difference in the thermodynamic properties of Ca^{2+} binding may reflect the difference in the overall conformational changes of the proteins. It is interesting to see whether the structures of binding sites of the pump protein show any similarity to those of other Ca^{2+}-binding proteins and which class of the Ca^{2+}-binding proteins it belongs to. In this context the thermodynamic nature of the reduction in the affinity for Ca^{2+} in the *E-state conformation is also of interest.

c) Sources for large entropy changes. Although quantitative assessment of enthalpic and entropic contributions to the free-energy changes for individual steps of the Ca^{2+}-pump cycle is not yet possible, it can be concluded that several intermediate reactions are characterized by large changes in entropy. Especially phosphoenzymes, formed either by the interaction with ATP in the presence of Ca^{2+} or with P_i in the absence of Ca^{2+}, are stabilized by a large increase in entropy.

One possible source of such an entropy gain is a loss of water structure as a conformational change in an enzyme molecule or a conformational rearrangement within its oligomeric structure, which takes place when the protein-bound acyl phosphate is formed at the catalytic site. Indeed, an involvement of hydration and dehydration phenomena at the protein-water interface of the Ca^{2+}-ATPase has recently been suggested (52, 53, 56, 62).

De Meis et al. (56) found that phosphorylation of the enzyme is greatly facilitated in a dimethyl sulfoxide (DMSO) and water mixture. De Meis (52) proposed the hypothesis that the catalytic site is more hydrophobic in the absence of Ca^{2+}, favoring P_i and the β-carboxyl group of the aspartyl residue to react to form the acyl phosphate bond spontaneously. The reaction would proceed as if in a gas phase, where the hydrolysis of acyl phosphates would be unfavorable, as indicated by large positive ΔH values calculated on a theoretical basis (117; see sect. IV). The major effect of DMSO is to increase the hydrophobicity of the medium and thereby further favor the phosphorylation. Agreeing with this hypothesis, the van't Hoff enthalpy of phosphoenzyme formation, i.e., the reversal of the hydrolysis in the presence of 40% (v/v) DMSO, is strongly exothermic ($\Delta H = -85$ kJ/mol and $\Delta S = -270$ $J \cdot K^{-1} \cdot mol^{-1}$ at pH 6.0) (59). This is a remarkable contrast to the thermodynamics of phosphorylation by P_i without DMSO (IX*E2*). In this hypothesis, some water molecules should be released from the catalytic site on phosphorylation of the enzyme by P_i. Dupont and Pougeois (62) made a detailed analysis of the water-activity dependence of the stability constant of the phosphoenzyme and obtained a result indicating that as many as 18 water

molecules are released from the catalytic site on phosphorylation. In agreement with this observation they also found that the phosphorylation is accompanied by a decrease of the polarity in the catalytic site.

The phosphoenzyme formed is of course the ADP-insensitive form. To confer ADP sensitivity on it, reduction of the DMSO concentration is necessary together with addition of Ca^{2+} (56). This result indicates that water participates in the transition from the *E − P to the E ~ P state. Although the role of this water is not known, one possibility is that the water would enter the catalytic site on *E − P to E ~ P transition. This view is consistent with the observation that the polarity of the catalytic site of the ADP-sensitive phosphoenzyme (formed by ATP) is not much different from that of the free enzyme (62).

Note, however, that such a difference in the polarity of the catalytic site between the *E − P and E ~ P forms does not necessarily negate the involvement of water-molecule movement in the formation of the latter phosphoenzyme in the normal catalytic cycle. For example, occlusion of Ca^{2+} bound to the high-affinity transport sites of the enzyme, which accompanies the E ~ P formation, must involve certain changes in the interaction of protein groups with water. Another possibility for hydration or dehydration to be involved would be changes in the interaction between the ATPase molecules, which exist as an oligomer (see sect. IX*B3*), during the catalytic cycle (222).

X. GENERAL FEATURES OF BIOLOGICAL ENERGY TRANSDUCTION

This review concentrates on the mechanism and energetics of the two major energy-transducing ATPases in muscle, actomyosin of the contractile machinery and the Ca^{2+} pump of SR membrane, which controls the activity of the actomyosin. Besides these muscle ATPases, ATPases of other energy-transducing systems essential for physiological functions of cells have been studied intensively in recent years.[9]

On the basis of reaction mechanisms, these ATPases are conventionally classified into either the actomyosin or Ca^{2+}-pump type. During the ATPase cycle, an enzyme-product (ADP + P_i) intermediate dominates in the former, as does the phosphoenzyme intermediate in the latter. The dynein-tubulin system belongs to the actomyosin group (265, 276, 277). This system is the

[9] Prof. Y. Tonomura, who died on November 28, 1982, was a pioneer in studying the mechanisms of ATPase reactions in biological energy transduction. We should appreciate that much of the recent progress in this area originated from his idea, which has been developed over the last three decades, when his first paper concerning the kinetics of actomyosin ATPase reaction was published (279).

molecular entity responsible for chemomechanical energy transduction by flagelli in eukaryotic organisms.

On the other hand, in a number of ion-transporting systems of cell membranes and intracellular organelles, phosphorylated forms of the ATPase proteins play a key role in chemiosmotic energy transduction. An intriguing exception for the membrane-bound ATPases is the ATP-synthetase systems in mitochondria, chromaffin granules, chloroplasts, and bacteria (7, 220), which utilizes the proton-electrochemical gradient across the membrane to produce ATP from ADP + P_i. In these systems, a myosin · ADP · P_i–like intermediate is formed rather than phosphorylated intermediates (107, 196, 197, 241).

Apart from such a chemical difference in the major reaction intermediate between these ATPase types, they share several common features that are probably directly relevant to the energy-transduction mechanisms. In the final section of this review, some of the salient points are emphasized, mainly based on the present analysis of the muscle ATPases, but also referring to other ATPases where appropriate.

A. Involvement of Specific Ligands and Their Translocation in ATP Hydrolysis

A fairly complex and highly ordered piece of apparatus is a prerequisite for any energy-transducing processes (303). In biological energy transduction this is required so that an ATPase molecule interacts with specific partner molecules or ions in such a way that chemical potential is not merely dissipated as heat but rather converted into a useful work. Here these molecules and ions are referred to as ligands. For example, actin and Ca^{2+} are ligands for myosin and the Ca^{2+}-pump ATPase, respectively. The essential structural requirement for the ATPase is that they have a specific site for binding a ligand that is distinct from the catalytic site where ATP hydrolysis occurs. Thus the catalytic and ligand sites are physically separated, and their interaction is mediated by distortion of a peptide chain within an ATPase molecule and/or change if any in interaction of subunits.

From an enzymological point of view, these ligands are enzyme activators. In the absence of their respective ligands, ATPases only catalyze the hydrolysis of their substrate slowly. Usually an ATPase has high affinities for its specific ligands. A crucial point, however, is that to fulfill its role as an energy transducer, the ATPase should change affinities for ligands in a cyclic way during the catalytic cycle. For example, the Ca^{2+}-ATPase shows a high affinity for Ca^{2+} to bind the cation at a low concentration on one side of the SR membrane (the myoplasm) but subsequently reduces its affinity to dissociate them into the other side where the cation level is much higher. The effect of such affinity changes is the translocation of Ca^{2+} across the SR membrane. A similar principle may operate in the H^+-translocating ATPase

system, where the molecular complex of ATPase is disposed across the membrane, which separates two aqueous phases with different proton-electrochemical potentials.

An actin filament may be regarded as a linear track of ligands. During muscle contraction the myosin molecules take states with low and high affinities for actin alternately and tend to associate with and dissociate from actin in a cyclic fashion. Thus myosin and actin filaments slide past one another if muscle is allowed to shorten or produce isometric force if not allowed.

Thus it seems a general property of energy-transducing ATPases to have intrinsic affinities for their ligands, which are changed during the ATP hydrolysis cycle to translocate ligands. A plausible hypothesis is that the affinities are changed at the expense of the chemical potential of ATP. To be more specific, however, it is necessary to introduce the basic concept that the ATPase can inherently assume two distinct conformational states that differ from one another in the properties of the ligand-binding site and the catalytic site.

B. Alternation Between Fundamental Conformational States

Spectroscopic and other structural evidence indicates that protein conformational changes do occur during the catalytic cycle of energy-transducing ATPases. An orthodox view is that such conformational changes are induced by chemical changes at the catalytic site, and a unique conformational state corresponds to a specific intermediate state of the enzyme.

On the basis of recent studies of the ATPase and some other enzyme systems, however, it seems more plausible that the ATPase proteins exist in two fundamental conformational states that are alternated during the energy-transduction process; changes in the state of ligand-binding sites are functional manifestations of these conformational states. A hypothesis is proposed here that their relative population is governed by chemistry of the catalytic site and occupancy of the ligand site. On one hand, a chemical change will result in a redistribution of the state population. The catalytic cycle is composed of a series of chemical changes. Some of these changes cause significant redistributions of the state population (at least two of which are in opposite direction for a cycle to be completed), but others do not. The interaction of the ATPase with ligands is then stabilized and destabilized in a cyclic manner during the ATPase cycle. Actomyosin (sect. VIII) and Ca^{2+} pump (sect. IX) are good examples.

On the other hand, if a ligand stabilizes one of the two conformational states of the ATPase protein that is not predominant in the catalytic cycle in the absence of the ligand, its binding could result in the acceleration of the catalytic cycle, provided that ATP reduces the affinity for the ligand. This is the activation of ATPase by a ligand.

Thus the hypothesis provides a basis for explanation of the indirect link between the catalytic event and ligand translocation and hence for energetic coupling of chemical and physical processes.

C. Energetics of ATPase Cycle

In the proposed hypothesis a series of chemical changes accompanying ATP hydrolysis at the catalytic site induces conformational transitions of the ATPase protein that bring the ligand sites into different orientations and affinities in a cyclic fashion. For the myosin-ATPase reaction, some of these chemical events and accompanying changes in protein conformation can now be characterized in thermodynamic terms. In short, the major free-energy change in ATP hydrolysis is the binding of ATP to myosin, whereas the free-energy change for the hydrolysis of myosin-bound ATP is negligible. The rest of this review is a discussion of how far these characteristic features of myosin-ATP hydrolysis can be generalized among various ATPases in reference to their implication in the energy-transducing mechanisms.

1. Hydrolysis of ATP on enzyme

Hydrolysis of ATP into ADP + P_i under physiological conditions is a typical exergonic reaction, and its major drive is the decrease in enthalpy. In contrast with the situation in free aqueous solution, the hydrolysis of ATP on myosin is virtually isoenergetic; i.e., the intermediate E · ATP and E · (ADP + P_i) are in equilibrium. Direct kinetic evidence has recently been obtained that this also applies to the F_1-ATPase reaction (107).

It is obvious that energy release on hydrolysis of free ATP does not take place when the terminal phosphate of ATP is split at the catalytic site of these ATPases. It is most likely that E · (ADP + P_i) is a key transit form through which the energy is switched from chemical to physical form or vice versa. In muscle contraction, ATP is bound to myosin with an extraordinarily high binding constant. This may be referred to as energy trapping. On hydrolysis of the enzyme, the trapped energy is converted into the transit form, which is then used to produce force when the cross-bridge transition occurs, probably from A · M · ADP · P_i to the A · M state. On the other hand, in synthesizing ATP on energy-transducing membranes where F_1-ATPase is the machinery to make an anhydrous bond between P_i and ADP, energy need not be supplied to convert E · (ADP + P_i) into E · ATP. Thus, once ADP and P_i bind together to the catalytic site of F_1, a protein-bound ATP is formed almost spontaneously. The complex is as stable as the myosin-ATP complex, and energy input is required to dissociate the complex, which is just the opposite of the energy trapping in myosin/ATP interaction (30, 237).

What occurs in ATPases that form phosphorylated intermediates? For the Ca^{2+}-pump ATPase, the actual step in which the protein-bound acyl

phosphate is formed by transfer of the terminal phosphate of ATP to a specific aspartyl residue

$$Ca_2 \cdot E \cdot ATP \rightleftharpoons Ca_2 \cdot E \sim P \cdot ADP$$

is also in equilibrium (see ref. 224). Accompanying this reaction, Ca^{2+} bound to the high affinity sites of the ATPase are brought into a state that is occluded from the bulk aqueous phase of either side of the membrane. When the phosphoenzyme is transformed into another form (*E − P), the Ca^{2+} sites are exposed again, but this time to the other side of the membrane, and the affinity is reduced. This step of Ca^{2+}-pump function is critical to the translocation of the cations across the membrane, and probably is the energy-transducing step. Thus the phosphoenzyme with occluded Ca^{2+} can be regarded as the transit form of the energy-transduction process. In the Na^+-K^+-ATPase system, the free-energy change for phosphoenzyme formation from the E · ATP is also suggested to be very small (88).

2. *High-entropy state of transit forms in biological energy transduction*

One of the most important contributions made by calorimetric studies of muscle ATPases is that the isoenergetic hydrolysis of ATP on myosin is endothermic. This is of considerable significance because it implies that the M · ADP · P_i intermediate is stabilized by a large increase in entropy. In other words, the transit form in the chemomechanical energy-transduction process is in a high-entropy state, whereas the strong binding of ATP to myosin and hence energy trapping is driven solely by a large decrease in enthalpy.

It is important to know if the E · ADP · P_i intermediate is also stabilized by entropy gain in the process of ATP synthesis by F_1-ATPase. On the other hand, formation of the phosphoenzyme with occluded Ca^{2+} by interaction of the Ca^{2+}-ATPase with ATP has been shown to be endothermic by calorimetry. Although further calorimetric studies are required to resolve steps involved in the phosphoenzyme formation, evidence suggests that the transit form of ion transport, at least in the Ca^{2+} pump, is also in a high-entropy state.

3. *Nature of transit state with high entropy*

Again in the case of myosin ATP hydrolysis, it has been suggested that there is a large loss of ordered water structure around the hydrophobic residues of the protein with isoenergetic transition from M · ATP to M · ADP · P_i (sect. VIII*B*). It is still too early to identify which parts of the myosin-water interphase are mainly dehydrated. An attractive speculation is that the catalytic site becomes more hydrophobic on ATP hydrolysis. As suggested by theoretical calculation, energetics of hydrolysis of so-called high-energy compounds in a hydrophobic environment is different from that

in an aqueous environment in that the equilibrium between reactants and products would not be much favored for the latter. Thus the high-energy phosphate ester bond could be made spontaneously in hydrophobic environments. This is obviously a very attractive idea that could explain the spontaneous formation of ATP on F_1-ATPase; i.e., energy input is not needed for formation of ATP itself but for its release.

In this context, note that the Ca^{2+}-pump ATPase and Na^+-K^+-ATPase are phosphorylated by P_i under appropriate conditions. This is a typical example of spontaneous formation of high-energy acyl phosphate bond. In the case of Ca^{2+}-pump ATPase the reaction occurs in the absence of Ca^{2+} as

$$*E + P_i \rightleftharpoons *E \cdot P_i \rightleftharpoons *E - P$$

(Note that Mg^{2+} is essential for this reaction but for simplicity has not been written.) Thermodynamic analysis indicates that formation of $*E \cdot P_i$ is accompanied by a rather large favorable free-energy change ($\Delta G < 0$) and is driven by a decrease in enthalpy. In contrast, the free-energy change over the acyl phosphate-formation step is almost nil ($\Delta G \simeq 0$), which is endothermic and hence driven by an increase in entropy. Thus, from a thermodynamic point of view, the reaction sequence is analogous to that for the interaction between myosin and ATP. An important point is that an increase in the medium hydrophobicity caused by adding certain organic solvents such as DMSO strongly facilitates the phosphorylation. This is also the case for ATP synthesis on F_1-ATPase (241). In addition, evidence, although indirect, has been obtained for the increase in hydrophobicity of the catalytic site of the enzyme by releasing water during phosphorylation. Because the reaction is the reversal of phosphoenzyme breakdown in the Ca^{2+}-pump cycle, hydrophobicity of the catalytic site must increase at some step or steps preceding the breakdown in the forward mode of operation of the pump. The most likely step is the formation of phosphoenzyme with occluded Ca^{2+}.

Thus there is a general possibility that hydration and dehydration at the catalytic site is a crucial mechanism for the formation and decomposition of the transit state through which the energy is transformed between chemical and physical forms during biological energy transduction.

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