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Distinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL

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The chaperonin GroEL is a double-ring structure with a central cavity in each ring that provides an environment for the efficient folding of proteins[1–3] when capped by the co-chaperone GroES in the presence of adenine nucleotides[4–8]. Productive folding of the substrate rhodanese has been observed in *cis* ternary complexes, where GroES and polypeptide are bound to the same ring, formed with either ATP, ADP or non-hydrolysable ATP analogues[2,9], suggesting that the specific requirement for ATP is confined to an action in the *trans* ring that evicts GroES and polypeptide from the *cis* side[9]. We show here, however, that for the folding of malate dehydrogenase and Rubisco there is also an absolute requirement for ATP in the *cis* ring, as ADP and AMP-PNP are unable to promote folding. We investigated the specific roles of binding and hydrolysis of ATP in the *cis* and *trans* rings using mutant forms of GroEL that bind ATP but are defective in its hydrolysis. Binding of ATP and GroES in *cis* initiated productive folding inside a highly stable GroEL–ATP–GroES complex. To discharge GroES and polypeptide, ATP hydrolysis in the *cis* ring was required to form a GroEL–ADP–GroES complex with decreased stability, priming the *cis* complex for release by ATP binding (without hydrolysis) in the *trans* ring. These observations offer an explanation of why GroEL functions as a double-ring complex.

The monomeric protein rhodanese has recently been shown to reach native form inside *cis* ternary GroEL–GroES complexes that were formed in ATP, AMP-PNP or ADP, albeit at different rates (ATP > AMP-PNP > ADP)[2,9]. The single-ring GroEL mutant SR1 has been used to produce obligate and stable *cis* complexes for study[1,2,9]. Because it has no second ring, the SR1 mutant does not receive the signal from *trans*-sided ATP that normally evicts GroES and substrate[6]. After GroES and any of the three nucleotides were added to rhodanese–SR1 binary complexes, productive folding was shown to occur in the *cis* cavity. We observed that rhodanese that was refolded inside SR1–GroES formed in the presence of ATP could be released efficiently to the medium by brief incubation at 4 °C, a treatment previously shown to lead to rapid dissociation of GroES from an ADP complex with GroEL[6]. Similarly, folding of ornithine transcarbamylase (OTC) from binary complexes with SR1 occurred in the presence of GroES, with almost identical kinetics with either ATP or ADP, when GroES was released by incubation at 4 °C, thereby allowing OTC trimerization (data not shown). These data suggest that the previous observation of OTC folding in the presence of ATP in a single turnover from a *cis* ternary complex[1] resulted from ADP-driven folding during preparation of the complex, with subsequent release when ATP was added. In experiments using SR1 and the green fluorescent protein (GFP), which also refolds inside *cis* complexes formed with any of the three nucleotides, brief treatment at 4 °C also leads to efficient release of GFP (Fig. 1a).

When the same tests were performed with two stringent substrate proteins, Rubisco from *Rhodospirillum rubrum*[10,11] and mitochondrial malate dehydrogenase (MDH) from pig heart[12–14], we observed that only ATP could promote reactivation from wild-type or SR1 *cis* ternary complexes (Fig. 1b, c). Release of GroES from SR1 by treatment at 4 °C was essential for production of enzymatic activity, because both Rubisco and MDH are homodimers, requiring assembly of the refolded monomeric subunits to reach active form. Direct incubation at 4 °C of metastable intermediates of Rubisco that were produced after dilution from denaturant (into the same chloride-free buffer used in all of the Rubisco studies)[6] did not result in enzymatic activity (data not shown). Remarkably, the kinetics of reactivation by the SR1–GroES ternary complexes in ATP were similar to, if not faster than, those achieved in similar reactions with wild-type GroEL, indicating that a stable folding-active state is produced at SR1. Addition of 'trap' molecules (such as 337/349)[15], which are able to bind but not release non-native substrate proteins, at the time of cold release of GroES had no effect on the kinetics of reactivation (kinetics not shown, but see Fig. 1f, traces 1, 4). These data indicate that, as with rhodanese, folding of both Rubisco and MDH proceeded to a committed state in the ATP *cis* ternary complexes; that is, the substrates reached conformations no longer recognizable by chaperonin.

We wished to follow directly the folding of substrate within the various *cis* complexes, thereby obviating any requirement for the release of GroES and peptide to assay enzymatic activity. We therefore examined by stopped-flow changes in the fluorescence total intensity and anisotropy of tryptophan residues of Rubisco in complexes formed following the addition of GroES and nucleotides to Rubisco–chaperonin binary complexes (Fig. 1d, e). This takes advantage of the absence of tryptophan from both GroEL and

GroES, and so allows us to monitor the conformational changes occurring in the substrate during the chaperonin reaction[16–18]. Addition of ATP and GroES to complexes of either SR1–Rubisco or wild-type GroEL–Rubisco resulted in an initial rapid drop in total fluorescence intensity and anisotropy ($t_{1/2} \sim 1$ s), followed by a

steady rise in both intensity and anisotropy ($t_{1/2} \sim 3.5$ min) (Fig. 1d, e; only anisotropy data are shown). The rate of the rising phase corresponded within a factor of two with the rate of recovery of Rubisco enzymatic activity from both SR1 and wild-type GroEL (Fig. 1b). The early decrease in anisotropy probably indicates the
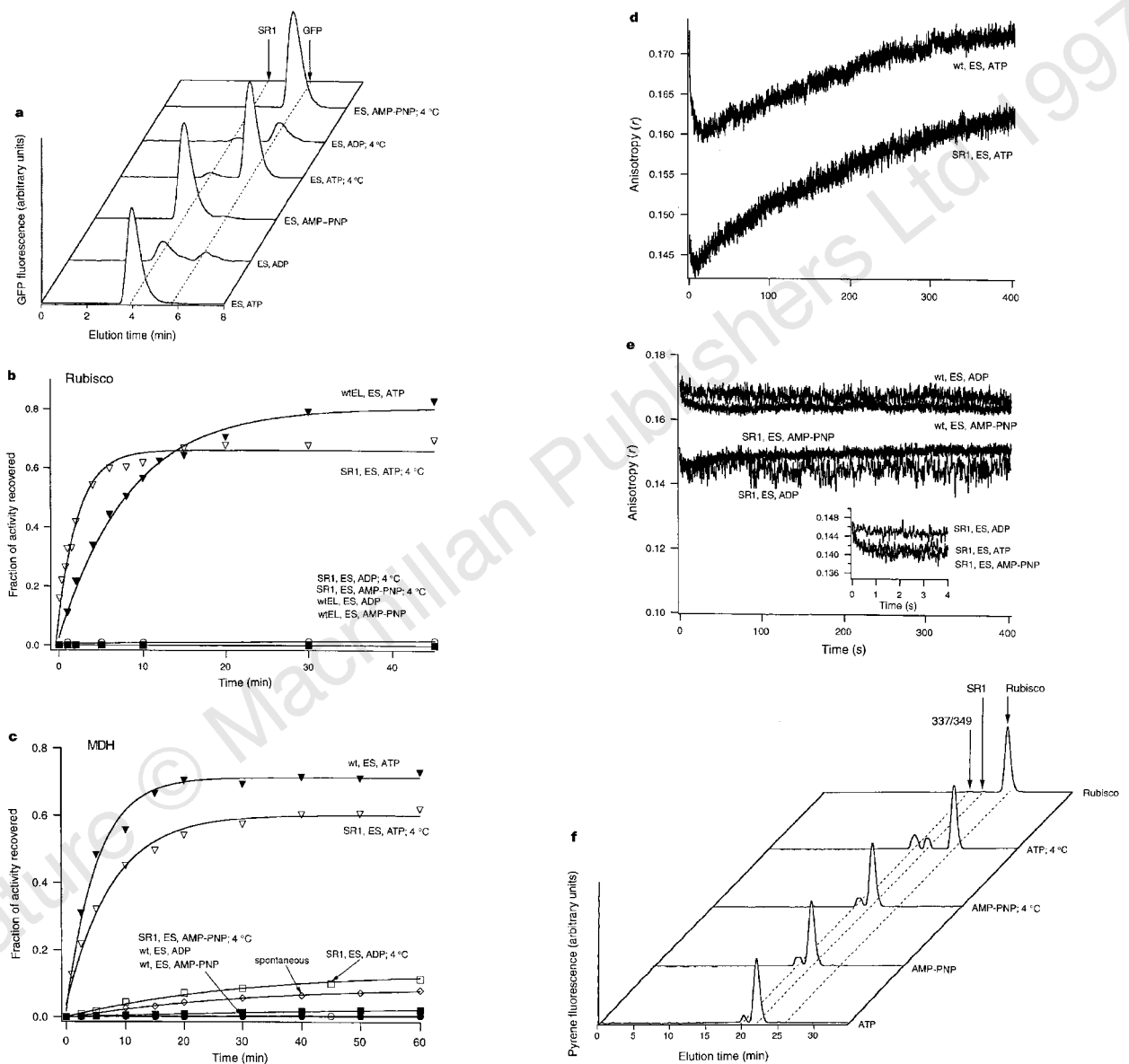

Figure 1 ATP is required in *cis* for the GroEL–GroES-mediated folding of two stringent substrates, Rubisco and MDH. **a**, Folding of the non-stringent substrate GFP inside the complex formed between the single-ring mutant, SR1, and GroES, and release of folded GFP by 4 °C treatment. Binary complexes between acid-denatured GFP and SR1 were prepared and mixed with GroES and either ATP, ADP or AMP-PNP. Samples of the complexes, with folded, trapped GFP inside, were incubated for 10 min at either 23 °C (unlabelled) or 4 °C, then subjected to gel filtration with on-line fluorescence detection. **b**, Refolding of Rubisco by wild-type GroEL and SR1. Binary complexes were formed between acid-denatured Rubisco, and either wild-type GroEL (wtEL) or SR1, and GroES (ES) and nucleotide were added. SR1 reactions were incubated at 4 °C for 15 min before enzyme assay to release bound GroES and free folded Rubisco monomers. SR1 reactions incubated at 23 °C demonstrated no Rubisco activity. Incubation of acid-denatured Rubisco for various times at 4 °C without chaperonin resulted in a recovery of only 1–2% activity. Total recovery is based on the activity of 100 nM native Rubisco (monomer). **c**, Refolding of mitochondrial MDH by wild-type GroEL and SR1. Refolding of MDH in binary wild-type GroEL–MDH or SR1–MDH complexes was initiated by the addition of GroES and nucleotide. The spontaneous recovery of refolded MDH in the absence of chaperonins was approximately 10%. Assaying SR1 reactions for enzymatic activity without prior incubation at 4 °C resulted in <4% recovery of activity. **d**, **e**, Folding of Rubisco with wild-type GroEL and SR1 monitored by changes in intrinsic tryptophan fluorescence anisotropy. Binary complexes were formed between acid-denatured Rubisco and either wild-type GroEL or SR1 and rapidly mixed (1 : 1) in a stopped-flow apparatus with solutions containing GroES and either ATP (**d**), ADP or AMP-PNP (**e**). The anisotropy of the Rubisco intrinsic tryptophan fluorescence was monitored as a function of time after mixing. Traces represent summations of 25 individual experiments (**d**) or 10–15 experiments (**e**). Inset, early time measurements with SR1; each trace is the sum of 40–50 experiments. **f**, Failure of Rubisco to fold in SR1–GroES–AMP-PNP complexes correlates with inability of the polypeptide to dissociate from SR1. Binary complexes were formed between acid-denatured RUBpyr and SR1 and mixed with GroES and either ATP or AMP-PNP. After 1 h at 23 °C, 337/349 GroEL trap tetradecamer was added, and the mixture incubated at either 23 °C or 4 °C for an additional 10 min before gel filtration. Based on proteolytic protection experiments (not shown), GroES binds as stably to the SR1–Rubisco complex in AMP-PNP as in ATP.
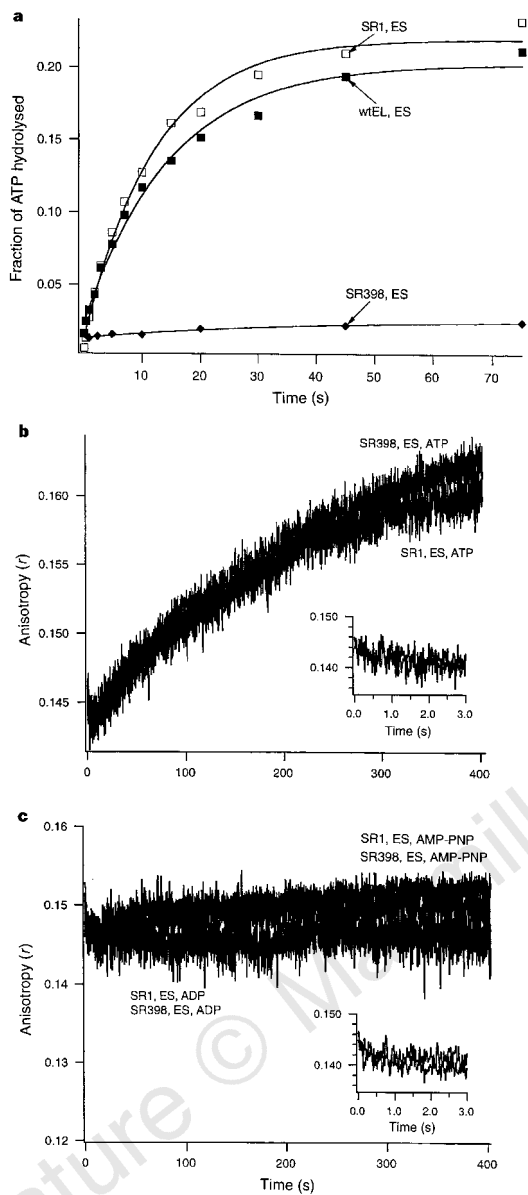
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Figure 2 Binding of ATP and GroES to the *cis* ring of GroEL is sufficient to trigger the release and folding of Rubisco. **a**, ATPase activity of wild-type GroEL (wtEL), SR1 and SR398 in the presence of GroES (ES) was measured by rapid mixing/quenching in a quench-flow apparatus. Experiments with binary complexes between each of these chaperonins and Rubisco showed no significant difference in hydrolytic activity. **b**, **c**, The SR398 ring is competent to fold Rubisco, despite absence of significant ATP hydrolytic activity. Binary complexes were formed between acid-denatured Rubisco and SR398 and rapidly mixed (1 : 1) in a stopped-flow apparatus with solutions containing GroES and either ATP (**b**), ADP (**c**) or AMP-PNP (**c**). Data for SR1 from Fig. 1 are shown again for comparison. Traces represent the summation of 25 (**b**) or 10–15 (**c**) experiments. Inset, early changes in anisotropy of the SR1 and SR398 complexes with ATP (**b**) and AMP-PNP (**c**). **d**, High affinity of GroES for SR398 in ATP, and recovery of refolded GFP and Rubisco from SR398 ternary complexes by proteolytic treatment. GFP–SR398 binary complexes were mixed with GroES and ATP and the mixture gel filtered at 23 °C with monitoring of fluorescence in-line. Exposure of the purified ternary complexes to 4 °C or 250 mM KOAc at 4 °C failed to significantly release the bound substrate. Exposure to 10% methanol or 0.4 M guanidine-HCl (GdHCl) led to only partial release. Treatment with trypsin followed by 4 °C incubation leads to full release of GFP. For recovery of refolded Rubisco from SR398–GroES–ATP ternary complexes, a similar treatment was performed using proteinase K followed by assay for enzymatic activity (inset).

rapid mobilization of reporting regions of the Rubisco polypeptide chain, associated with release of polypeptide from the apical binding sites. The slower increase in intensity and anisotropy, associated with recovery of activity, probably reflects the burial and packing of the tryptophan side chains into the core of the refolding Rubisco monomer. In contrast, these slow rising changes were not observed when ADP or AMP-PNP was substituted for ATP, correlating with the failure of these nucleotides to support production of the native state (Fig. 1e). However, AMP-PNP, but not ADP, induced the same initial rapid drop of both intensity and anisotropy that was observed with ATP (Fig. 1e, inset), suggesting that binding of GroES with AMP-PNP produces some of the same early local motion in Rubisco. However, given that no further anisotropy changes occurred (although there was a slow drop in intensity), it seems that the polypeptide is either not fully released or is recaptured by the apical peptide-binding surface[19].

The ongoing association of Rubisco with chaperonin in AMP-PNP was observed directly in an experiment with SR1 and RUBpyr, which is a pyrene-labelled derivative of Rubisco. RUBpyr bears a single pyrene that does not interfere with catalytic function and allows GroEL-mediated refolding to native form, which occurs as completely as with unmodified Rubisco but at a slower rate. As shown in the gel filtration profiles (Fig. 1f), RUBpyr was efficiently refolded when GroES and ATP were added to SR1–RUBpyr binary complex, because, after release with treatment at 4 °C, RUBpyr migrated to the position of the Rubisco homodimer. In contrast, however, when GroES and AMP-PNP were added to the binary complex, RUBpyr comigrated with SR1 after treatment at 4 °C, indicating that the non-native form remained bound to SR1, unable to be captured by added 337/349 trap (Fig. 1f). Thus both anisotropy and gel-filtration studies indicate that the formation of the *cis* complex in ATP releases Rubisco into the central channel, initiating folding, whereas formation of *cis* complex in AMP-PNP (or ADP) does not fully release the polypeptide. AMP-PNP does not seem to produce the same stereochemical changes in GroEL–GroES complexes as ATP[20], reflecting the fact that AMP-PNP is not a perfect ATP homologue.

These studies demonstrated that productive conformational changes of substrate protein were triggered when ATP and GroES were added, but it was not clear whether the immediate trigger to folding was initial ATP–GroES binding or subsequent ATP hydrolysis. To resolve this question, we examined a mutant in which Asp 398 in the intermediate domain was affected, a residue shown in the GroEL–GroES–ADP crystal structure to have moved from a remote location in the unliganded structure into the equatorial

nucleotide-binding site to a position just distal to the β-phosphate of ADP, and which contributes to the ligation of Mg^{2+} (ref. 19). Consistent with the structural analysis, the D398A mutant was able to bind ATP with normal affinity, but was defective in ATP turnover,

Figure 3 ATP hydrolysis in the *cis* ring 'primes' release of GroES and peptide ligands by *trans*-sided ATP. **a**, Schematic representation of the mixed-ring GroEL complex, MR2. The ring containing D398A mutant subunits can bind polypeptide and GroES but is defective in ATP hydrolysis; the 3-7-9 ring cannot bind polypeptide or GroES as a result of apical substitutions in its subunits, but is proficient in ATP hydrolysis[21]. Apical (A), intermediate (I) and equatorial (E) domains of the GroEL subunits are shown; X, mutations. **b**, ATP triggers release of GroES bound to MR2 in ADP but not in ATP. Gel filtration analyses are shown of chaperonin mixtures in which the single-ring GroES 'trap' SR398, was added to capture GroES released from binary complexes formed between MR2 and ^{35}S-radiolabelled GroES. Top, A_{229} profile of MR2 and SR398; middle panels, MR2–GroES complexes formed in either ATP or ADP do not release GroES. Bottom, addition of ATP triggers release of GroES from MR2–GroES binary complex formed in ADP. **c**, Rubisco refolded in MR2-GroES-ATP complexes can be productively released by addition of ATP after prolonged incubation of the purified ternary complexes. RUBpyr–MR2 binary complexes were incubated with GroES in the presence of ATP and the ternary complexes purified by gel filtration in the absence of nucleotide. Portions of ternary complex were exposed to ATP at the indicated time at 23 °C after initial mixing, and mixtures were fractionated by gel filtration with in-line fluorescence detection. Migration positions of MR2 and native homodimeric Rubisco are shown.

with ~2% wild-type activity (data not shown). As expected, the D398A tetradecamer bound non-native rhodanese and, in the presence of GroES and ATP, formed a complex that could mediate production of native rhodanese but could not release the protein (data not shown). Similarly, when this mutation was introduced into SR1, the derived SR398 complex bound ATP and GroES in the absence of significant hydrolysis (Fig. 2a) and refolded rhodanese in the ternary complex without releasing the native protein. Fluorescence-dynamics studies were also performed with SR398–Rubisco complexes. When GroES and ATP were mixed rapidly, total intensity and anisotropy changes were observed that were virtually identical to those seen with the hydrolysis-proficient SR1 complex (Fig. 2b, c). Once again, when AMP-PNP or ADP were used instead of ATP, the rising (productive) phase of anisotropy/intensity was absent, although AMP-PNP again promoted an early drop of anisotropy in SR398-bound Rubisco. We conclude that ATP–GroES binding alone is sufficient to trigger Rubisco release and productive folding in *cis* ternary complexes.

To determine whether Rubisco subunits could reach native form inside the SR398–GroES ternary complex, we sought to measure enzyme activity after incubation at 4 °C, as with SR1. Surprisingly, no activity was detected, owing to the failure of GroES to release from the SR398 complex. This failure was demonstrated by gel filtration of ^{35}S-GroES–SR398–Rubisco ternary complexes that had been exposed to incubation at 4 °C, where ^{35}S-GroES migrated with the complex (data not shown). This was further supported by gel filtration of SR398–GroES–GFP complexes in which, after incubation at 4 °C, the fluorescent refolded GFP remained associated with SR398 (Fig. 2d). This latter analysis indicated that even transient release of GroES, which would allow escape of GFP, did not occur. Remarkably, much more severe treatments, including incubation in 0.4 M guanidine HCl, also failed to release GFP from SR398–GroES–ATP (Fig. 2d). We finally resorted to partial proteolytic treatment with trypsin, which released intact, fluorescent GFP from the chaperonin complex (Fig. 2d). When a similar proteolytic step was performed with Rubisco bound in SR398–GroES–ATP, we observed consistent recovery of significant activity (Fig. 2d; 4–6% in each of three independent experiments). Significantly, the same low level of recovery was observed after proteolysis of the productive SR1 ternary complex (from which >60% activity was recovered if 4 °C release was used instead, as in Fig. 1b). The low recovery in both cases presumably results from unintended proteolytic digestion of refolded substrate protein. These findings support the indications from fluorescence studies that binding of ATP and GroES is sufficient to trigger folding of at least a fraction of Rubisco molecules to native form in the SR398 complex.

The high affinity of GroEL for GroES in ATP revealed by the study of hydrolysis-defective D398A mutants suggested that, under normal conditions, the *cis* ring must undergo hydrolysis of bound ATP before ATP in the *trans* ring can trigger GroES release. The ADP-bound state presumably comprises a lower-affinity GroEL–GroES interaction, or is more responsive to a signal for release of GroES produced by the *trans* ring. To test this, we produced a mixed-ring GroEL assembly[21], composed of a D398A ring, which was able to bind polypeptide and GroES but unable to hydrolyse ATP, and a 203/337/349 (3-7-9) ring, which was unable to bind substrate or GroES but able to provide normal ATP function in *trans* to the D398A ring (see Fig. 3a). The mixed-ring complex, MR2, was tested for ability to stably bind ^{35}S-labelled GroES in ATP or ADP. In the presence of ATP, MR2 stably bound GroES, failing to release it even though there was hydrolysis in the 3-7-9 ring (at a rate ~21% of wild-type, unaffected by GroES). In particular, when SR398 was added as a GroES 'trap' to a mixture containing MR2–GroES and ATP, no transfer of radiolabelled GroES from MR2 to SR398 was observed by gel-filtration analysis (Fig. 3b). Thus the high affinity for GroES of the D398A ring of MR2 in ATP seems to recapitulate that observed with SR398 in ATP. GroES was also stably bound to
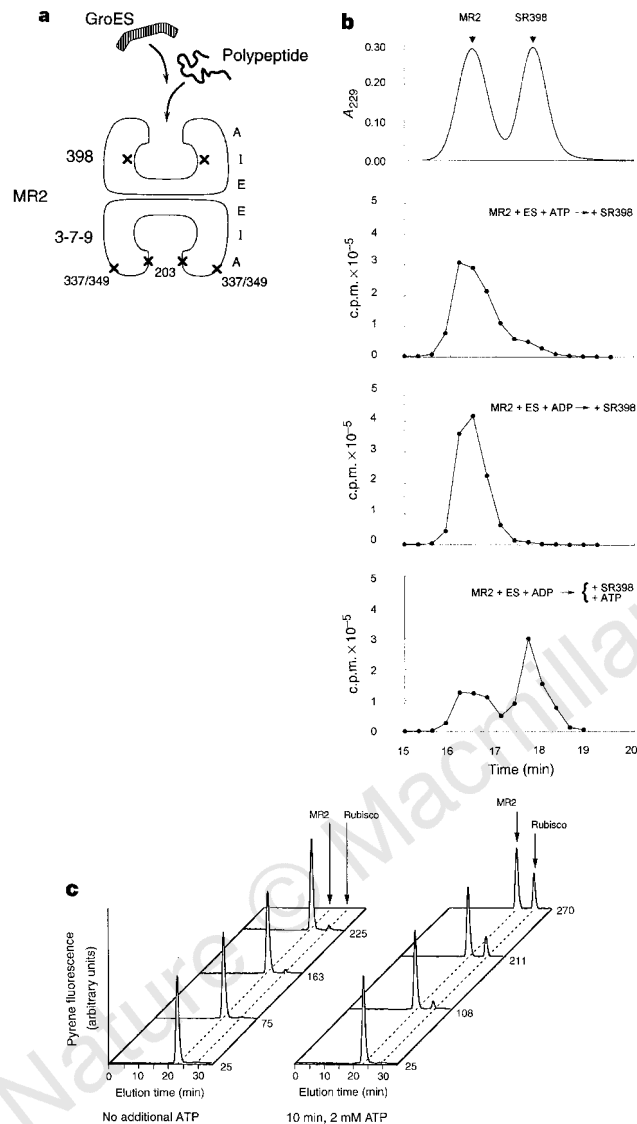
MR2 in ADP, although if ADP was absent from the gel filtration running buffer, the GroES was released (data not shown). This would seem to be consistent with an apparently lower affinity interaction of GroES with the D398A ring in ADP (compared with no release from the ATP complex when it was gel filtered in the absence of nucleotide). More importantly, however, when the MR2–GroES complex formed in ADP was exposed to ATP, GroES was released efficiently, and was detected through its capture by co-incubated SR398 (Fig. 3b). This suggests that the pre-bound ADP *cis* complex emulates the post-hydrolysis ADP *cis* complex in being similarly coupled to the action of *trans*-sided ATP, allowing GroES to be released.

The consequences of *cis* ATP hydrolysis for release of folded polypeptide were examined more directly by forming RUBpyr–MR2–GroES complexes in ATP, purifying them by gel filtration, and incubating them in the absence of nucleotide for prolonged periods, during which the bound ATP slowly hydrolysed in the D398A ring. When such complexes were then exposed to ATP and the reaction mixtures gel filtered, there was a progressive increase with incubation time in recovery of fluorescence at the position of native homodimeric Rubisco, reflecting the gradual priming of the *cis* ring for *trans*-ATP-driven release of the folded RUBpyr (Fig. 3c).

Once we had observed the specific requirement for binding of ATP in the *cis* ring to promote folding, with AMP-PNP unable to substitute, we then investigated whether a similar requirement might be operative in the *trans* ring with respect to driving GroES/polypeptide release from the *cis* side. Previous studies have observed that AMP-PNP and ATP-γS supplied in *trans* are unable to support the release of GroES from *cis*, but have been interpreted to indicate that hydrolysis is required in *trans* for eviction from *cis*[6]. To address this question, the hydrolysis-defective D398A tetradecamer was used to form folding-active *cis* ternary complexes in ATP which were allowed to slowly hydrolyse bound *cis* ATP over 2 h to produce a 'primed' *cis*-ADP state (as in Fig. 3c). The complexes were then incubated briefly with ADP, AMP-PNP or ATP. When GFP was examined as a substrate, gel-filtration studies showed that the folded protein remained stably associated in D398A ternary com-

plexes (purified away from the nucleotide) over the 2-h time period. GFP was not released upon subsequent addition of ADP or AMP-PNP (Fig. 4). However, when ATP was added, gel filtration after 2 min showed that >60% of the GFP was released (Fig. 4), which corresponds to the efficiency of GFP release observed when ATP was added to a wild-type *cis*-ADP–GFP complex (Fig. 4). MDH was also used as a substrate in a similar experiment (not shown), in which limiting amounts (just sufficient for formation of *cis* complex) of ATP were supplied, followed by incubation for 2 h. Once again, brief exposure to ATP but not ADP or AMP-PNP produced recovery of enzymatic activity, the apparent result of release of refolded monomer from the *cis* ternary ADP complex only by ATP. These studies with D398A indicate that binding of ATP in *trans*, independent of hydrolysis, is sufficient to promote the release of GroES and polypeptide from *cis*-ADP complexes.

In summary, our results indicate that GroEL-mediated folding of stringent substrates like Rubisco and MDH requires action of ATP in both the *cis* and *trans* rings (Fig. 5). In the *cis* ring, binding of ATP and GroES triggers folding, potentially beginning within 1 s, a finding that is in accord with the early and rapid drop of anisotropy reported here and elsewhere[2]. ATP, specifically, promotes the complete release of stringent substrates like Rubisco into the central channel, which enlarges at least twofold in volume in association with GroES binding and changes in surface character from hydrophobic to hydrophilic[19] (Fig. 1d, f). Hydrolysis of ATP in the *cis* ring, occurring with a $t_{1/2}$ of 6–8 s (see Fig. 2a and ref. 7), is then necessary to relax the high-affinity interaction of GroES with ATP-bound GroEL, priming the ring for the release of GroES, which is accomplished by subsequent binding of ATP in the *trans* ring. □

Methods

Proteins. Tetradecameric GroEL with the D398A substitution (D398A) was produced by polymerase chain reaction (PCR)-mediated site-directed mutagenesis of a plasmid encoding wild-type GroEL[21]. A single-ring mutant containing the D398A substitution (SR398) was produced by exchanging a
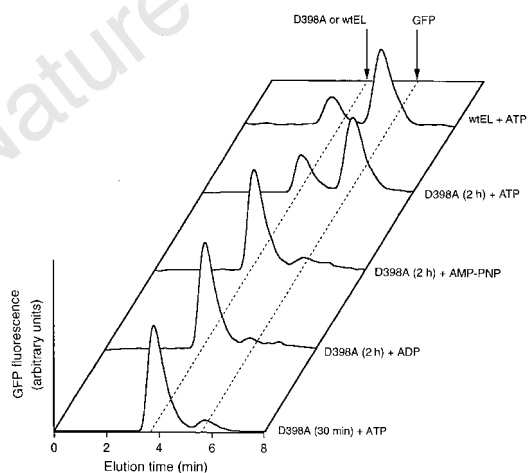

Figure 4 Release of GFP refolded in tetradecameric D398A *cis* ternary complexes by subsequent addition in *trans* of ATP but not ADP or AMP-PNP. GFP–D398A binary complexes were incubated with GroES and ATP, treated briefly with proteinase K to remove any GFP bound in *trans*, then purified by gel filtration in the absence of nucleotide. The complexes were then incubated for 30 min or 2 h, then exposed to ATP, ADP or AMP-PNP for 2 min and rapidly gel filtered with in-line fluorescence detection. By 2 h the amount of GFP released by ATP from D398A tetradecamer is similar to that obtained from GFP–wtGroEL–GroES–ADP *cis* complexes exposed to ATP for 1 min. ATP triggered release from D398A, but ADP and AMP-PNP had no effect.
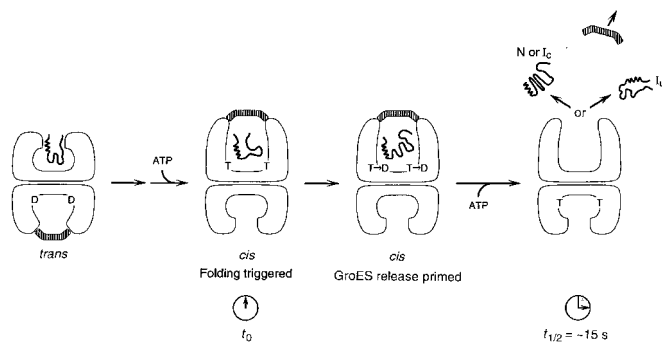

Figure 5 Schematic representation of the action of ATP in *cis* and *trans* rings during GroEL–GroES-mediated protein folding. Polypeptide is initially bound in the *trans* ring of a GroEL–GroES binary complex, and a step of ATP-triggered GroES release and rebinding or of binding of a second GroES to the polypeptide-bound ring occurs, forming a *cis* complex in which polypeptide is sequestered in the GroEL central channel underneath GroES[23,24]. The act of formation of the *cis* complex, through binding of ATP and GroES to the ring with bound polypeptide, triggers rapid release of polypeptide from the apical binding sites into the central channel, and folding begins (t_0) (see Figs 1, 2). Hydrolysis of ATP in the *cis* ring weakens the high-affinity interaction between GroES and (*cis*)ATP-bound GroEL (see Fig. 3), and binding of ATP in *trans* subsequently produces release of GroES and polypeptide from the *cis* ring ($t_{1/2}$~15 s) (see Fig. 4). D, ADP; T, ATP; T → D, hydrolysis of ATP; N, native; I_c, non-native conformation committed to completing folding to native form (no longer recognizable by chaperonin); I_{uc}, non-native form that must be rebound by chaperonin or by a different chaperone to reach native form.

restriction fragment with coding sequences bearing the D398A mutation for the wild-type segment with that in an expression plasmid encoding SR1. GroEL, SR1 and the derivatives, as well as GroES, were expressed in *Escherichia coli* and purified as described[1]. Metabolically labelled [^{35}S]GroES was produced as described[1]. Pig heart mitochondrial malate dehydrogenase (Sigma) was purified by gel filtration chromatography. Soluble, fluorescent GFP and Rubisco from *Rhodospirillum rubrum* were expressed at 20 °C from T7 promoters in BL21 transformants in the absence of induction and were purified by ion-exchange chromatography.

GFP refolding. All GFP folding experiments were conducted in buffer A (50 mM HEPES, pH 7.6, 5 mM KOAc, 10 mM Mg(OAc)$_2$, 1 mM DTT) at 23 °C. GFP ($\sim$530 μM) was acid-denatured by a 5-fold dilution with 25 mM glycine-phosphate buffer (pH 2.0). After 5 min at 23 °C, binary complexes were generated by diluting the denatured GFP 11-fold (9 μM final concentration) into 27 μM SR1 (Fig. 1a) or SR398 (Fig. 2d) in buffer A and incubating for 10 min at 23 °C. For SR1 experiments (Fig. 1a), GroES was added to 50 μM and either ATP to 1.5 mM or AMP-PNP to 5 mM, or GroES was added to 165 μM and ADP to 1.5 mM. Folding mixtures were incubated at 23 °C for 45 min. One portion was placed at 4 °C for 10 min, while another was maintained at 23 °C. All samples were then chromatographed over three Tosohaas TSK-gel guard columns connected in series. In-line fluorescence detection of renatured GFP used excitation at 339 nm and an emission cut-off filter centred at 495 nm. For SR398 experiments (Fig. 2d), GroES was added to 66 μM and ATP to 2 mM, and the mixture was incubated at 23 °C for 15 min, then gel filtered on the guard columns. The purified ternary complex was either reinjected or first treated for 15 min with the indicated conditions. For protease treatment, one portion was supplemented with trypsin to 15 μg ml^{-1} and incubated at 23 °C for 10 min. Soybean trypsin inhibitor was then added to 125 μg ml^{-1}, the sample incubated at 4 °C for 10 min, and then injected. For D398A (tetradecamer) studies (Fig. 4), ternary complex was formed as above. After 15 min at 23 °C, proteinase K was added (1 μg ml^{-1} final concentration) to remove GFP bound in *trans*. After 5 min, PMSF was added to 1 mM and the complex was purified by gel filtration on the tandem guard columns in buffer A. The purified complex was incubated in the dark at 23 °C for 30 min or 2 h. Samples were then supplemented with either ATP (2 mM), ADP (5 mM) or AMP-PNP (5 mM), incubated for 2 min, then reinjected to the guard columns and analysed by on-line fluorescence detection. Wild-type GroEL–GroES–GFP complex was prepared similarly, except that ADP (1 mM) and GroES 150 μM were used. Despite the low yield of this assembly, the ADP-ternary complex was stable until challenged with ATP, as determined by rechromatographing.

Rubisco refolding. Rubisco was denatured by a 1:55 dilution into 25 mM glycine-phosphate buffer, pH 2.0. Immediately after a 3–5 min incubation at 23 °C, binary complexes were formed by mixing the unfolded Rubisco with chaperonin in a total volume of 1.5 ml buffer A. The final concentrations were 130 nM wild-type GroEL, 300 nM SR1 or SR398, and 100 nM Rubisco (monomer). Folding was initiated by addition of a two-fold molar excess of GroES (over chaperonin) and either ATP (1 mM), ADP (5 mM) or AMP-PNP (5 mM) (final concentrations). For wild-type reactions, at the times indicated, ATP was removed by adding hexokinase and glucose (final concentrations, 0.08 U μl^{-1} hexokinase and 10 mM glucose). For SR1 reactions, ATP was removed 30 s after its addition by adding hexokinase and glucose. SR1 reactions were incubated at 4 °C for 15 min before enzyme assay to release bound GroES and free folded Rubisco monomers. SR1 samples were briefly allowed to re-equilibrate to 23 °C before enzyme assay. For proteolytic release of Rubisco from SR398–GroES and SR1–GroES ternary complexes (Fig. 2d), after 7 min of refolding at 23 °C (see Fig. 1b) the mixture was supplemented to 3 mM Ca(OAc)$_2$ and proteinase K was added to 125 μg ml^{-1}. After incubation at 23 °C for 6 min, PMSF was added to 3 mM and the mixture assayed after a further 10 min at 23 °C for Rubisco activity. Rubisco enzyme activity was determined by incorporation of [^{14}C]CO$_2$ into acid-precipitable products[10].

MDH refolding. All MDH refolding studies were performed in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM MgCl$_2$, 10 mM DTT (buffer B). MDH was denatured for 1 h in 5 M urea in buffer B at 30 °C. Binary complexes were formed between wild-type GroEL or SR1 and MDH by rapidly diluting the denatured MDH 250-fold using a solution of 1.2 μM wild-type GroEL or 5 μM

SR1 in buffer B such that the final concentration of MDH was 1 μM with respect to subunits. Refolding was initiated by the addition of a 3-fold molar excess of GroES and either 2 mM ATP, 10 mM ADP or 10 mM AMP-PNP, followed by incubation at 30 °C. At various times a portion was withdrawn and, in the case of wild-type GroEL, assayed immediately for enzymatic activity. In the case of the SR1-mediated reaction, the ATP was removed by hexokinase and glucose as before, then incubated at 4 °C for 10–15 min before assay to release bound GroES and free folded, assembly-competent MDH monomers. For the experiment with D398A, binary complex was formed with 5 μM MDH subunit and 7 μM D398A oligomer (100 μM subunit). GroES and ATP were added to 10 μM and 50 μM final concentrations, respectively. After 2 h at 30 °C, proteinase K was added at 800 ng ml^{-1}, and incubation carried further for 5 min. PMSF was then added to 1 mM. Samples were then supplemented with ATP, AMP-PNP or ADP to 4 mM and after 90 s were assayed for MDH activity. MDH enzyme assay was performed by diluting a portion from the folding assay into 1 mM ketomalonate, 0.2 mM NADH, 10 mM DTT, and 50 mM Tris-HCl, pH 7.4, and following the oxidation of NADH at 340 nm (ref. 14).

Stopped-flow fluorescence anisotropy. The stopped-flow fluorescence anisotropy apparatus used in these studies was constructed by H.S.R. using as a template a design from the laboratory of J. Beechem[16]. It consists of a Bio-Logic SFM/4 stopped-flow head coupled to an excitation source and mono-chromator (Photon Technologies International) through a fibre-optic bundle designed to provide a small slit-shaped (3 × 0.66 mm) excitation beam to the stopped-flow cuvette (FC.15). The two emission channels are collected in T-format through a pair of non-fibre, large core diameter, high numerical aperature (NA 0.59) light guides (Oriel) placed directly against the stopped-flow cuvette to maximize light collection efficiency. Polarization selection of the excitation and emission used film polarizers (Meadowlark Optics) placed over the end of each light guide at the stopped-flow cuvette. The collected fluorescence is spectrally filtered and detected with a pair of Hammamatsu PMTs (R4457P) operated in photon-counting mode. Data collection was as previously described[16]. For stopped-flow experiments involving Rubisco, a sample was diluted (1:9) with 25 mM glycine-phosphate buffer, pH 2.0, to produce a 15.6 μM acid-denatured Rubisco stock. Each binary complex was generated by diluting 480 μl of this stock into 5 ml of buffer A containing either wild-type GroEL (2 μM), SR1 (4 μM) or SR398 (4 μM). After 10 min at 23 °C, the binary mixture was loaded into one syringe of the stopped-flow apparatus. The binary mixture was rapidly mixed (1:1) in the stopped-flow (at 23 °C) with a solution containing GroES (6 μM) and either ATP (2 mM), ADP (10 mM) or AMP-PNP (10 mM).

Quenched-flow ATPase assay. ATPase activities of wild-type GroEL, SR1 and SR398 were determined using a quenched-flow rapid mixing apparatus (KinTek) at 20 °C. Equal volumes (15 μl) of chaperonin in buffer A and GroES/[γ-^{32}P]ATP in buffer A were mixed to give final concentrations of 2.85 μM chaperonin, 6 μM GroES, 100 μM [γ-^{32}P]ATP (300 μCi μmol^{-1}), then the reaction was quenched with 67 μl 1.5 M formic acid/1.5 mM potassium phosphate at the times indicated. Note that the ATP concentration is such that hydrolysis by wild type is not at steady state after one turnover. Portions of each sample were chromatographed on polyethylene imine thin-layer plates, developed with 1 M formic acid and 0.25 M LiCl. Radioactivity in phosphate and ATP regions was quantified by phosphorimager analysis.

Labelling of native Rubisco with pyrene (RUBpyr). Rubisco (0.8 mg ml^{-1} in 100 mM sodium phosphate, pH 7.6) was reduced with 2 mM TCEP and labelled with a 6–7-fold excess of *N*-1-pyrene-maleimide, added in two portions. The reaction was quenched by adding glutathione (2 mM), and the labelled Rubisco was separated from unbound dye by gel filtration (PD-10 column, Pharmacia). The labelling ratio was 1:1, pyrene to Rubisco monomer, as determined by absorption spectroscopy, using extinction coefficients of 67,000 M^{-1} cm^{-1} at 280 nm for the Rubisco monomer[22] and 36,000 M^{-1} cm^{-1} at 340 nm for reacted *N*-1-pyrene-maleimide (Molecular Probes). Labelling of a single cysteine (usually the surface-accessible Cys 58) was indicated by detec-tion of a single major tryptic peptide with strong absorption at 340 nm during separation by C8 reverse-phase chromatography. The specific activity of the labelled enzyme was indistinguishable from that of unlabelled Rubisco, and refolding of acid-denatured RUBpyr was dependent upon GroEL, GroES and

ATP, although renaturation occurred at a rate approximately half that of the unlabelled enzyme.

Refolding and gel filtration with RUBpyr. For RUBpyr refolding, the labelled enzyme was denatured in acid glycine-phosphate buffer as described above. For the experiment shown in Fig. 1, binary complexes were formed by incubating denatured RUBpyr with SR1 in buffer A (final concentrations, 2 μM RUBpyr and 4 μM SR1) for at least 10 min at 23 °C. GroES was added to 5 μM, and folding was initiated by adding either ATP to 1 mM or AMP-PNP to 5 mM. After 1 h at 23 °C, 337/349 GroEL trap was added to 6.5 μM and incubation continued at either 23 °C or 4 °C for a further 15 min. All samples were then subjected to gel filtration on a TSK G-4000SW$_{xl}$ column equilibrated in buffer A. For the experiment shown in Fig. 3, a 20-μM solution of unfolded RUBpyr was mixed with buffer A containing MR2 to give final concentrations of 4 μM RUBpyr and 5 μM MR2. After 10 min at 23 °C, GroES was added to 10 μM and ATP to 2 mM, and the mixture incubated at 23 °C for 5 min. Unbound ATP was removed by rapid (4 min) gel filtration over three TSK guard columns connected in series and equilibrated in buffer A. The purified MR2–RUBpyr–GroES complex was incubated at 23 °C for the times indicated, then 200-μl portions of the complex were either directly injected onto the G-4000 gel filtration column equilibrated in buffer A (to assess the intrinsic stability of the complex), or were supplemented with ATP to 2 mM for 10 min, then gel filtered. For all experiments, pyrene fluorescence was detected with an in-line fluorescence detector with excitation at 339 nm and an emission long-pass filter centred at 470 nm.

Production of mixed-ring complex and GroES binding. Mixed-ring complex MR2 was produced and purified as described previously for MR1 (ref. 21), with incubation for at least 1 h of the parent tetradecamers, D398A and 3-7-9 in MgATP at 42 °C. For studies of GroES binding to MR2, ^{35}S-GroES (40,000 c.p.m. pmol^{-1}) was incubated at a final concentration of 0.3 μM with 1 μM MR2 and either ATP or ADP (1 mM) in 10 mM KCl, 25 mM Tris-HCl, pH 7.4, 1 mM MgCl$_2$. After 20 min at 20 °C, SR398 was added to 2 μM and, where indicated, ATP was added to a final concentration of 10 mM. After 5 min further incubation, the mixtures were chromatographed on a G4000SW$_{xl}$ column in the same buffer containing 1 mM ADP. Fractions were collected and counted directly.

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DNA renaturation activity of the SMC complex implicated in chromosome condensation

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Chromosome condensation occurs in mitosis before the separation of sister chromatids, and requires DNA topoisomerase II (refs 1, 2) and a group of proteins called SMCs[3–5]. The resulting condensed chromosomes in metaphase have a complex hierarchical structure[6,7]. SMCs, the components of condensed chromosomes, are also required for the separation of sister chromatids and gene dosage compensation, and are found in a range of organisms from yeasts to mammals[8–13]. However, the mechanisms by which the SMCs contribute to chromosome condensation are unknown. We have studied chromosomes in fission-yeast SMC mutants *cut3-477* and *cut14-208* (ref. 9), which remain largely non-condensed during mitosis at the restrictive temperature (36 °C)[9]. To test their role in DNA condensation, we isolated the proteins Cut3 and Cut14 as an oligomeric complex, and tested their interactions with isolated DNA. The complex efficiently promoted the DNA renaturation reactions (the winding up of single-strand DNAs into double helical DNA) as much as ~70-fold more efficiently than RecA[14], which is a bacterial protein with similar activity. The activity of the mutant complex was heat sensitive. As DNA winding by renaturation is a potential cause of supercoiling, the SMC complex may be implicated in promoting the higher-order DNA coiling found in condensed chromosomes.

To obtain isolated Cut3 and Cut14 proteins from *Schizosaccharomyces pombe* cells, six-histidine tags were attached and they were simultaneously overproduced using the inducible promoter REP1 (ref. 15) (Fig. 1a). Cells were collected and lysed, and the extract put over a Ni-NTA column[16]. Fractions containing Cut14–HA6His (relative molecular mass 137,000 (M_r 137K)) and Cut3 (155K) at 80% purity were detected, indicating that the two proteins formed a complex[10] in overproduced cells (Fig. 1b, c). The complex was further purified by phosphocellulose P11.

After sucrose-gradient centrifugation[17], a broad distribution of Cut3–Cut14 at equal proportions was obtained at around 7–13S (the peak was at 13S; Fig. 1d, top). Gel filtration showed that the complex had an M_r of 600K or larger (data not shown). The purified Cut3–Cut14 complex thus seemed to exist as a heterotetramer or possibly a larger complex, but a smaller form (heterodimer) might also exist. A similar oligomeric complex was also isolated from extracts of cells that were not overproduced (Fig. 1d, bottom).

The purified complex was found to renature DNA extremely efficiently (Fig. 2). Denatured, single-stranded linear DNAs were mixed with the complex, proteins were extracted, and the resulting