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The reaction of GroEL (cpn 60) with the ATP analogue 2′,3′ dialdehyde ATP

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The reaction of the *E. coli* chaperonin GroEL (cpn 60) with the ATP analogue 2′,3′ oxidised ATP (oATP) has been studied. Treatment with the reagent leads to loss of the ATPase activity of GroEL in a pseudo-first-order fashion; this can be prevented by inclusion of ATP in the reaction mixture. Measurements of the stoichiometry of the reaction indicate that the loss of activity corresponds to the incorporation of about one oATP per subunit of GroEL. From analysis of the sequences of modified peptides it is proposed that the reaction probably occurs with one or both of the two cysteines Cys-457 and Cys-518, although the instability of the adduct(s) makes a definite identification of the site(s) of reaction difficult. The involvement of Cys side chains in the reaction with oATP was confirmed by using Nbs_2 (5,5′-dithiobis(2-nitrobenzoate)) to estimate thiol groups in both modified and unmodified GroEL.

GroEL protein; Chaperonin 60; Affinity labelling; 2′,3′ dialdehyde ATP; ATP binding site; Electrospray mass spectrometry

1. INTRODUCTION

Molecular chaperones are a group of proteins which assist in the folding, translocation and assembly of proteins in the cell [1,2]. Chaperonins form a subclass of molecular chaperones of which two are encoded by the *gro*E gene of *E. coli*. These proteins GroEL, a tetradecamer of subunit M_r 57,200 and GroES, a heptamer of subunit M_r 10,200 were discovered as critical components for the assembly of large bacteriophages such as lambda and T4 [3,4]. They are the subject of a great deal of current research aimed at elucidating the mechanism by which they assist in the correct folding and assembly of other proteins [5–7]. GroEL is known to possess a weak ATPase activity ($k_{cat} \sim 0.1\ s^{-1}$ per GroEL subunit) and it has been demonstrated that ATP binding is related to protein folding and/or release of polypeptide chains from the GroEL protein (usually in conjunction with its co-chaperonin GroES). The effect of K^+ and GroES on the rate of hydrolysis has been investigated in detail [5,8] and the results interpreted in terms of a model involving the asymmetric behaviour of the two heptameric rings of GroEL.

Many ATP binding proteins possess sequence motifs such as the Walker et al. type A motif [9], often abbreviated to -G-X_4-G-K-T/S-. Some members of the chaperonin 60 class show the presence of an extended Walker type A motif which contain an extra two amino acids between the first and second glycine residues of the consensus sequence [10]. The reported cDNA derived amino acid sequence of GroEL [11] does not contain any such sequence, although Lewis et al. [12] have defined a conserved -GDGTT- sequence as being a putative ATP binding site. In order to define amino acid residues involved in ATP binding we have investigated the use of the ATP analogue, 2′,3′ dialdehyde ATP (oATP) [13] as a possible affinity label.

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2. EXPERIMENTAL

GroEL was purified from *E. coli* strain DH1 carrying either the plasmid pND5 [14] or pGT3270 [15] as previously described [16]. The quantitation of the protein was performed as previously described using a value of 0.285 for the specific absorption coefficient (litre · g^{-1} · cm^{-1}) at 280 nm [17].

ATPase activity of GroEL was measured by a coupled enzyme assay procedure as described [16], except that a buffer of 50 mM triethanolamine pH 8.0 containing 10 mM magnesium acetate was used.

oATP was synthesized by oxidation of ATP by sodium periodate as described [13]. Solutions of oATP were aliquotted and stored at −70°C before use. [^{3}H]oATP was synthesised by periodate oxidation of [2,5′,8-^{3}H]ATP. The final specific activity of the product was 55,500 dpm/nmol [^{3}H]oATP.

The reaction of oATP with GroEL was performed at 25°C in a buffer of 50 mM triethanolamine pH 8.0 containing 10 mM magnesium acetate (unless otherwise stated). After inactivation of GroEL, a 10-fold excess of sodium borohydride over oATP was added to quench the reaction.

The mass of the GroEL modified peptide was determined on a VG BioTech Platform single quadrupole mass spectrometer operating in the electrospray ionisation mode. Spectra were acquired in both positive and negative ion mode.

3. RESULTS AND DISCUSSION

3.1. *Inactivation of GroEL ATPase activity in the presence of oATP and demonstration of substrate protection*

The ATPase activity of GroEL was found to be approximately 0.1 s^{-1} per subunit, in close agreement with other reported values [5,8]. As shown in Fig. 1A, the inclusion of oATP with GroEL led to a decrease in activity in a pseudo-first-order fashion. Using 1 mM oATP the half life of inactivation was 56 min, with essentially all ATPase activity lost after 6 h incubation. The rate of ATPase inactivation was concentration dependent with respect to oATP (data not shown). In the absence of oATP there was no loss in activity over time and inclusion of ATP led to substantial protection against inactivation (Fig. 1A,B). This indicates that inactivation is likely to be due to interactions at or near the ATPase active site.

In preliminary experiments it was found that addition of freshly thawed oATP solution to GroEL resulted in a lag period of approximately 2 h before any substantial inactivation occurred. This lag could be eliminated by preincubation of the oATP at 25°C for 2 h before addition. However preincubation of GroEL at 25°C for 2 h before the addition of freshly thawed oATP did not abolish the lag. The time course of inactivation and protection by ATP was unchanged whether or not sodium borohydride was added.

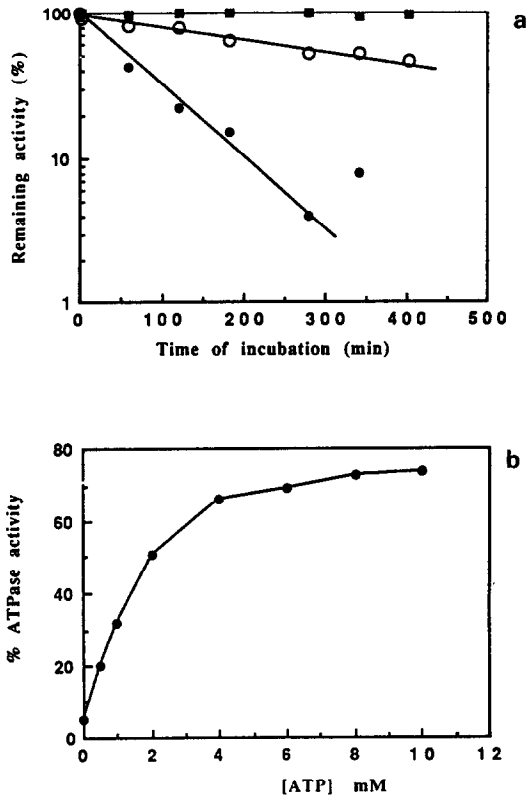

Fig. 1. (a) Inactivation of GroEL ATPase activity by oATP. GroEL (0.3 mg/ml) was incubated at 25°C with 1 mM oATP in the absence (●) and presence of 4 mM ATP (○). The incubation buffer contained 50 mM triethanolamine pH 8.0 and 10 mM magnesium acetate; samples were assayed for ATPase activity at the times indicated. (■) shows GroEL ATPase activity at 25°C in the absence of oATP. (b) Protection of ATP against oATP inactivation. GroEL (0.3 mg/ml) was incubated for 6 h in the presence of 1 mM oATP, under the conditions described, and variable concentrations of ATP. After 6 h samples were assayed for activity.

3.2. *Stoichiometry of incorporation of oATP*

The stoichiometry of incorporation of oATP into GroEL was studied using [^{3}H]oATP. After inactivation and sodium borohydride treatment the protein was precipitated, in the presence of bovine serum albumin as a carrier protein, by the addition of 10% trichloroacetic acid and the pellet washed exhaustively in 10% trichloroacetic acid to remove unbound [^{3}H]oATP. The pellet was then solubilised in 1 M NaOH before measuring incorporated radioactivity. It was found that when GroEL has been 95% inactivated it had approximately 1.8 mol of [^{3}H]oATP incorporated per subunit. When the inactivation was carried out in the presence of 5 mM ATP (where only 30% activity had been lost) the extent of incorporation was considerably smaller corresponding to approximately 0.7 mol of [^{3}H]ATP per GroEL subunit. This indicates that the inactivation of the ATPase activity of GroEL is due to the incorporation of about 1 oATP per subunit, which can be protected by ATP, although additional reactions appear to occur at other sites. Thus by the criteria of protection by substrate and stoichiometry of modification it appears as though oATP is acting as an affinity label for the ATPase site of GroEL.

3.3. *Effect of oATP on proteinase K treated GroEL*

It has been reported that treatment of GroEL with proteinase K leads to the removal of a portion of the C-terminal part of the polypeptide chain, including the highly conserved G/M rich last 13 residues [18]. The truncated GroEL retains approximately only 20% of its ATPase activity but maintains its characteristic tetradecameric structure and is able to function in mediating protein folding with rhodanase as a substrate. This finding is consistent with the observations of McLennan et al. [15] who prepared mutants of GroEL lacking the G/M rich segment.

When proteinase K treated GroEL was incubated with [^{3}H]oATP the extent of incorporation was essentially the same as for native GroEL (i.e. 2.2 mol oATP per truncated GroEL subunit). The extent of incorporation in the presence of ATP was also reduced in this truncated form of GroEL (0.9 mol oATP incorporated per subunit), suggesting that these particular C-terminal residues do not play an important role in ATP binding. In agreement with other workers our proteinase K treated GroEL lost 70% of its ATPase activity after a 60 min digestion with proteinase K (2% w/w) at 25°C.

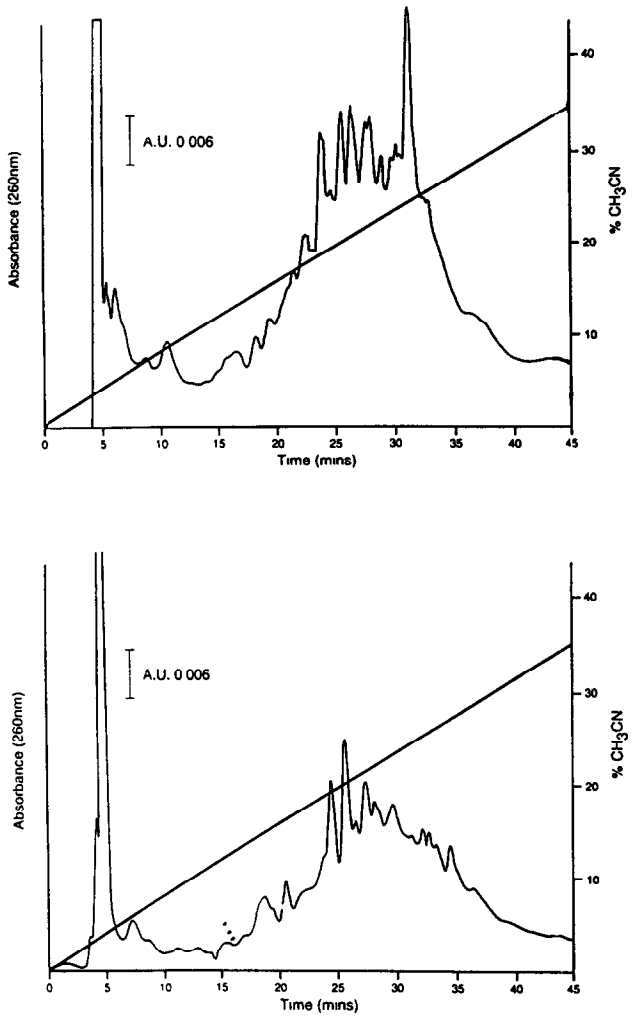

Fig. 2. Separation of peptides obtained by the thermolytic digestion of oATP modified (upper) and ATP protected GroEL (lower) by reverse-phase chromatography on a Waters C18 μBondapak column. Digestion was carried out as described, and peptides were eluted from the column by a linear CH_3CN gradient and monitored at 260 nm.

3.4. *Identification of the site(s) of modification by oATP*

In order to determine the site(s) of modification of GroEL by oATP, 5 mg of GroEL was reacted with 1 mM oATP for 6 h by which time less than 5% of the ATPase activity remained. A parallel experiment was performed in the presence of 5 mM ATP. Both reaction mixtures were quenched by the addition of sodium borohydride and subjected to gel filtration on Sephadex G-25 (equilibrated in 0.5% ammonium bicarbonate) to remove excess reagent. The 2 samples of GroEL were then digested with 3% (w/w) thermolysin for 24 h at 37°C. The peptides produced were subjected to reverse phase HPLC using a μBondapak C-18 column. When monitored at 214 nm the complex peptide profiles were sufficiently similar to make identification of a modified peptide very difficult. However, when monitored at 260 nm to detect adenine containing peptides the profiles were clearly different (Fig. 2) with a peptide eluting after 32 min (25% CH_3CN) present in the modified GroEL but absent in the protected sample.

Material from this peak was subjected to Edman degradation in the gas phase sequencer at the SERC Protein Sequencing Facility, Aberdeen. Two principal peptide sequences were found, namely:

$_{453}$ **IVLNXGEEPS**
$_{514}$ **ITTEX**

with X corresponding to Cys-457 and Cys-518, respectively, in the GroEL sequence. These peptides occurred in approximately equal amounts but it has not proved possible to resolve the HPLC peak at 32 min into separate peaks. When subjected to mass spectroscopy analysis the material in the modified peak consisted of a major component (approximately 95% of the material) of mass 1623, which would correspond to the 2 peptides above linked by a disulphide bridge between the Cys residues.

From these results it would appear that the modification of GroEL by oATP occurs in the regions corresponding either to residues 453–462 and/or residues 514–518 in the GroEL sequence. This raises a number of intriguing questions which we are at present unable to resolve completely. In the peptide sequence determined there are no Lys residues, which have been proposed as the usual site of modification of oATP [19]. The only plausible site(s) of reaction are the Cys side chains present in the 2 peptides. Reaction of the aldehyde group(s) of oATP at either Cys-457 or Cys-518 (or a cross link of the oATP with the 2 Cys residues) would presumably yield thiohemiacetyl derivatives. These derivatives appear to be stable under the conditions used for HPLC and digestion, but are apparently unstable under conditions used for mass spectroscopy analysis and Edman degradation. Under these conditions it would appear that the adenine moiety is lost and that the presence of the 2 Cys containing peptides could lead to the formation of a disulphide bridge. Clearly a more detailed investigation of the chemistry of the reaction and the stability of the derivatives is required to clarify this situation.

Direct evidence for the involvement of cysteine side chains in the reaction between GroEL and oATP was obtained by estimating the available thiol groups using Nbs_2 [20]. When 250 μM Nbs_2 was added to 0.2 mg/ml GroEL in 50 mM sodium phosphate buffer pH 8.0 at 25°C, there was no increase in A_{412} over a 30-min period. However, when the experiment was repeated in the presence of 0.1% SDS, the increase in A_{412} corresponded to the reaction of 3.1 ± 0.17 thiol groups per GroEL subunit. Thus in the native protein the 3 Cys residues known to be present in each polypeptide chain from the amino acid sequence [11] are clearly unavailable for reaction, presumably being buried. When GroEL was reacted with 1 mM oATP for 14 hours (under which

conditions > 95% ATPase activity was lost), the thiol content was reduced to 1.0 ± 0.14 per chain. Again this remaining thiol was only available for reaction after the addition of 0.1% SDS. Preliminary experiments have demonstrated that ATP protection results in an increased availability of the thiol groups for Nbs_2 reaction. It is thus clear that reaction of GroEL with oATP leads to the loss of thiol groups, consistent with the results of the sequencing work above.

The Cys residues of GroEL are not conversed so presumably Cys-457 and Cys-518 are not essential for the ATPase catalytic function of the protein; however they are nucleophilic groups which can react with the oATP. The indication that Cys-457 and/or Cys-518 may form part of the ATP binding site is consistent with a number of observations. Alignments of the amino acid sequences of members of the chaperonin 60 proteins (including the TCP-1 class) show 2 regions of high homology [12]. A conserved feature of these proteins is ATP hydrolysis, so comparisons of sequences should help to indicate regions involved in this function. The first block of homology between amino acids 85–95 show a highly conserved motif centered around -GDGTT-. Lewis et al. [12] have identified this as a putative ATP binding domain closely related to that in cAMP dependent protein kinase. The second region of homology in these proteins is observed between residues 400–500, where there are no significant deletions or insertions, which contain a conserved -GGG- motif. This region may also play an important role in binding parts of the ATP molecule.

Recently, Girshovich and co-workers have shown that [^{35}S]ATP-γS can form cross links to Cys-137 in GroEL. Analysis of the stability of single and double mutants in which Cys residues are replaced by Ser has shown that Cys-518 is in close proximity to Cys-137 (Girshovich et al. unpublished results). Although the detailed 3D structure of GroEL is not yet known, our results taken together with those of others indicate that the tertiary structure of each subunit must be such as to bring these regions of the polypeptide chain into close proximity so as to create the ATP binding site. It should be noted that the reactive functional groups of oATP are in the ribose moiety of the adenine nucleotide. One possible arrangement is that the N-terminal half of GroEL is involved in the binding of phosphate groups of ATP, as has been observed in a number of ATP binding proteins [9,21], whilst the amino acids near the C-terminus are involved in the binding of the sugar moiety. It remains a task for future work to fully characterise the amino acids involved in the ATP binding site of GroEL.

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