GroES and the chaperonin-assisted protein folding cycle: GroES has no affinity for nucleotides

Matthew J. Todd[a], Olga Boudkin[b], Ernesto Freire[b], George H. Lorimer[a,*]

[a]*Dupont Co. Experimental Station, Central Research and Development Department, Wilmington, DE 19880-0402, USA*
[b]*Biocalorimetry Center, Department of Biology, The Johns Hopkins University, Baltimore, MD 21218, USA*

Received 31 December 1994

Abstract **The *E. coli* chaperonin proteins, GroEL and GroES, assist in folding newly synthesized proteins. GroES is necessary for GroEL-assisted folding under conditions where the substrate protein cannot spontaneously fold. On the basis of photolabelling of GroES with 8-azido-ATP, a role for nucleotide binding to GroES in chaperonin function was suggested [Martin, et al., *Nature*, 366 (1993) 279–282]. We confirm the photolabelling of GroES with 8-azido-ATP. However, other proteins not known to contain nucleotide binding sites also became photolabeled suggesting that labeling is non-specific. Using rigorous physical methods, isothermal calorimetry and equilibrium binding, no interaction between GroES and nucleotides could be detected. We conclude that GroES has no nucleotide binding site.**

Key words: Protein folding; Chaperone; GroES; Calorimetry; Nucleotide binding

1. Introduction

Chaperones are ubiquitous proteins which can assist in the proper folding of newly synthesized proteins [1], protect denatured proteins from accumulating during stress [2], and prevent aggregation [3]. *E. coli* has two chaperonin proteins, GroEL and GroES, which transiently form a complex in the presence of nucleotides [4,5]. Non-native proteins bound to GroEL are protected from aggregation, and in an ill-understood mechanism requiring Mg^{2+},K^+-ATP, and GroES, the unfolded proteins are released [6,7], allowing them a brief opportunity to fold before rebinding to GroEL.

Central to the mechanism of GroEL-assisted protein folding is the role of GroES. Both GroEL and GroES are toroidal proteins; GroEL consists of two rings with 7 subunits each, and GroES is a single ring of 7 subunits [4,8,9]. The GroES co-chaperonin is indispensable under conditions where a substrate protein cannot spontaneously fold [10]. One proposed function of GroES is to coordinate ATP hydrolysis at all seven sites on one GroEL toroid, lowering the affinity of that ring for unfolded polypeptides. Thus GroES would serve to 'quantize' the subunits in a GroEL ring [6]. Alternatively, Martin et al., based on affinity labeling of GroES, suggested that the co-chaperonin interacts directly with nucleotide, perhaps passing the nucleotide through to the GroEL chaperonin [11,12].

Using the conditions of Martin et al. [12] we confirm the photolabeling of GroES by 8-azido-ATP. However, we can detect no thermodynamic interaction between nucleotide and GroES. Under equilibrium conditions GroES shows no affinity for nucleotide (ATP, ADP, or 8-azido-ATP) even at concentrations in excess of physiological levels. Thus, we question the conclusion that GroES binds nucleotide and the physiological function such a conclusion implies.

2. Experimental

GroEL and GroES were purified as described previously [5]. Nucleotides were Mg-salts (Sigma); 8-azido-ATP and 8-azido-[α-^{32}P]ATP was purchased from ICN. The pH of all nucleotides was adjusted to neutrality before use.

Isothermal titration calorimetry experiments were carried out using Omega instrument (Microcal Inc., Northhampton, MA) coupled with Keithley 181 nanovoltmeter used as a preamplifier to improve the signal-to-noise ratio [13]. The amount of power required to maintain the reaction cell at constant temperature after each injection was monitored as a function of time. Experiments were carried out by injecting 5 mM ATP in 20 mM Tris-HCl, 100 mM KCl, 5 mM MgOAc, pH 7.8, in 10 ml increments into the reaction cell (cell volume: 1.4 ml) containing 20 mM GroES heptamer in the same buffer. Control experiments were run in the absence of GroES in order to correct for the effect of the dilution of the ligand. The heat due to the reaction between the ligand and the enzyme was obtained as the difference between the heat of the reaction and the corresponding heat of dilution. Analysis of the data was carried out as described previously [14]. Positive controls were performed with ribonuclease A (Ribonuclease I; EC 3.1.27.5; Sigma) in 15 mM potassium acetate buffer, pH 5.5, with 5 μl increments of 2 mM adenosine 2′,3′-cyclic monophosphate (sodium salt) into the reaction cell containing 100 mM protein.

Equilibrium nucleotide binding to chaperones was measured using ultrafiltration. The purified chaperonin proteins [5] were incubated (at 25°C) in the upper chamber of a tared 0.4 ml ultrafree MC filter unit (30 kDa; Millipore) in 20 mM Tris-HCl, pH 7.4, 5 mM $Mg(CH_3CO_2)_2$, 100 mM KCl, with the given nucleotide concentrations. These conditions are identical to those employed by Martin et al. [12]. After 30 min, samples were centrifuged at 4000 × *g* for 1 min (25°C), resulting in ~40% of the liquid in the filtrate, ~60% in the retentate. 10 μl of both filtrate and retentate were analyzed on a Biosil S-400 (Bio-Rad) gel filtration column, equilibrated in 25 mM NaP_i, pH 6.9, 50 mM NaCl, 0.5 mM $MgCl_2$, monitoring absorbance at 212, 260, and 280 nm. For some reactions, ^{32}P-labeled nucleotide was used, and the counts associated with filtrate and retentate determined. The absence of nucleotide in the protein peak was confirmed: (a) by monitoring the absorbance profile of protein off the column, which was identical to that incubated in the absence of nucleotide; and (b) by liquid scintillation counting when using labeled nucleotide. Chaperonin concentrations were calculated from the volume change, and determined directly by integration of chromatograms. The two values agreed within 10%. Membrane integrity was confirmed by noting the lack of the chaperonin in the filtrate.

3. Results and discussion

The role of GroES is central to the mechanism of chaperonin-assisted folding under non-permissive conditions. Martin et al. [12] recently suggested that GroES donates ATP to

*Corresponding author. Fax: (1) (302) 695-4509.

SSDI 0014-5793(95)00021-6

GroEL, thus increasing the cooperativity of ATP binding. Evidence for this role came from studies where GroES was photolabeled with 8-azido-ATP, or 8-azido-ADP. A Mg^{2+} requirement was cited as evidence for specificity, and a conserved tyrosine (Y71) was identified as the single site of modification on the basis of negative evidence: it was the only residue not detectable in a pool of heterogeneous radio-labeled peptides derived from both GroEL and GroES [12]. Given the instability of adducts formed from the nitrenes of photo-activated 8-azido-ATP, even the positive identification of a given residue, absent quantitative recovery, is hardly compelling evidence, negative evidence even less so. The unreliability of 8-azido-ATP as a photo-probe of nucleotide binding sites emerges from the recent crystal structure of the F_1-ATPase [14]. Of the residues previously believed to form the adenine-binding pocket on the basis of photolabeling with 8-azido-ATP [15] none is the active site. These considerations, in addition to our own experience with GroES, raised our skepticism. Accordingly, we have attempted to measure the interaction of GroES with nucleotides, using physically more rigorous equilibrium methods.

Isothermal titration calorimetry experiments were carried out on GroES from *E. coli* measuring the heat absorbed or released upon addition of nucleotide (Fig. 1) using the same conditions as reported previously [12]. The heat of ATP dilution, under the conditions described in Fig. 1A,B was 14.24 ± 0.65 μcal per injection in the presence of GroES, and 15.10 ± 0.68 μcal per injection in the absence of GroES. Typical enthalpy values for nucleotide binding to protein range from −16 kcal/mol for 3′-CMP and ribonuclease A to −12 kcal/mol for 3′-GMP and barnase [16,17]. The statistical identity of nucleotide dilution in the presence of GroES was not consistent with the co-chaperonin developing a stable interaction with nucleotide, detectable by calorimetry under the indicated conditions. An example of the anticipated protein–nucleotide interaction which gives off energy (exothermic) is demonstrated in Fig. 1C,D.

These results suggested that GroES has no affinity for nucleotide, yet labeled nucleotide analog was clearly incorporated into the co-chaperonin protein [12]. Therefore we repeated the labeling experiments (Fig. 2) using conditions described previously [12]. We confirmed GroES labeling; however, other proteins (BSA and rhodanese) were labeled to a similar extent (Fig. 2; quantitation not shown). Even contaminants in the commercial protein preparations and BSA dimers became heavily labeled. Neither BSA, nor rhodanese is known to contain a nucleotide binding site. The above results are most consistent with non-specific photolabeling of GroES due to the longevity and reactivity of the nitrenes generated upon photoactivation [18].

Table 1
Equilibrium nucleotide binding by chaperonins

Equilibrium condition	Nucleotide bound per protomer
7.1 μM $GroES_7$ + 100 μM ADP	<0.01
29 μM $GroES_7$ + 400 μM ATP	0.01
11 μM $GroES_7$ + 153 μM 8-N_3-ADP	0.04
5.7 μM $GroEL_{14}$ + 100 μM ADP	0.44
5.7 μM $GroEL_{14}$ + 200 μM ADP	0.96
5.7 μM $GroEL_{14}$ + 153 μM 8-N_3-ADP	1.05

Equilibrium condition reported refers to the initial concentration of chaperonin and nucleotide, before ultrafiltration. Buffer conditions are as described in section 2.

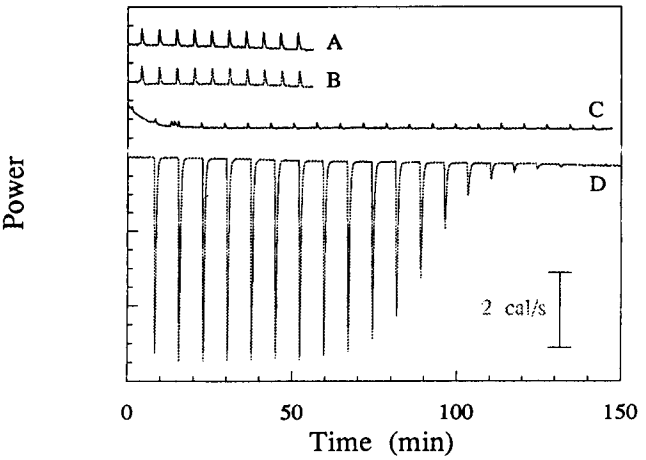

Fig. 1. A heat of nucleotide dilution in the presence/absence of GroES. Isothermal titration calorimetry was carried out by injecting 5 mM ATP in 20 mM Tris-HCl, 100 mM KCl, 5 mM MgOAc, pH 7.8, in 10 ml increments into the reaction cell (cell volume: 1.4 ml) containing 20 mM GroES heptamer in the same buffer (A). Control experiments were run in the absence of GroES (B) in order to correct for the effect of ligand dilution. The heat due to the reaction between the ligand and the enzyme was obtained as the difference between the heat of the reaction and the corresponding heat of dilution. Positive controls were performed with ribonuclease A (ribonuclease I; EC 3.1.27.5; Sigma) in 15 mM potassium acetate buffer, pH 5.5, with 5 μl increments of 2 mM 3′-CMP into the reaction cell (cell volume: 1.4 ml) containing 0 (C) or 100 μM protein (D). (Heats of dilution depend on the square of the amount of the ligand injected as well as on the physical properties of the ligand, thus the control in B has larger sized peaks than in C.)

In order to assess the significance of photolabeling of GroES by 8-azido-nucleotides, we measured the equilibrium binding of nucleotides to GroES. Chaperonin and nucleotide were incubated for 30 min, then a portion of the reaction was ultrafiltered using a 30 kDa membrane. An aliquot of both the filtrate and retentate were analyzed either by scintillation counting (when ^{32}P-labeled nucleotides were used) or by gel filtration HPLC, where any nucleotide associated with GroES dissociates and can be detected spectrophotometrically (Table 1). A comparison of the nucleotide concentrations in the retentate and the filtrate, whether determined radiometrically or spectrophotometrically, demonstrated that at equilibrium, GroES had no affinity for either ATP, ADP, or 8-azido-ADP. Identical reactions were run at pH 7.4 and 7.8, with 5 or 10 mM Mg^{2+}, and 0, 100, or 500 mM KCl. In no case was binding of nucleotide by GroES observed, whereas GroEL demonstrated half-site occupancy at 100 μM nucleotide and full-site occupancy by 200 μM nucleotide (Table 1). Thus under equilibrium conditions, GroES demonstrated no apparent affinity for nucleotide.

Whereas Martin et al. [12] used 60–100 nM $GroES_7$ and 1–5 μM 8-azido-nucleotide, our equilibrium binding experiments start with 7–25 μM $GroES_7$ and 100–400 μM nucleotide (Table 1). In some experiments, the final concentration of $GroES_7$ was in excess of 100 μM (data not shown). Since binding is proportional to the product of the concentrations of the two reactants, the results reported in Table 1 and Fig. 1 should favor nucleotide binding. The product of the reactant concentrations during these equilibrium studies approached physiological levels and was 3–4 orders of magnitude higher than those used previously [12]. An absence of specific binding is consistent with the ab-

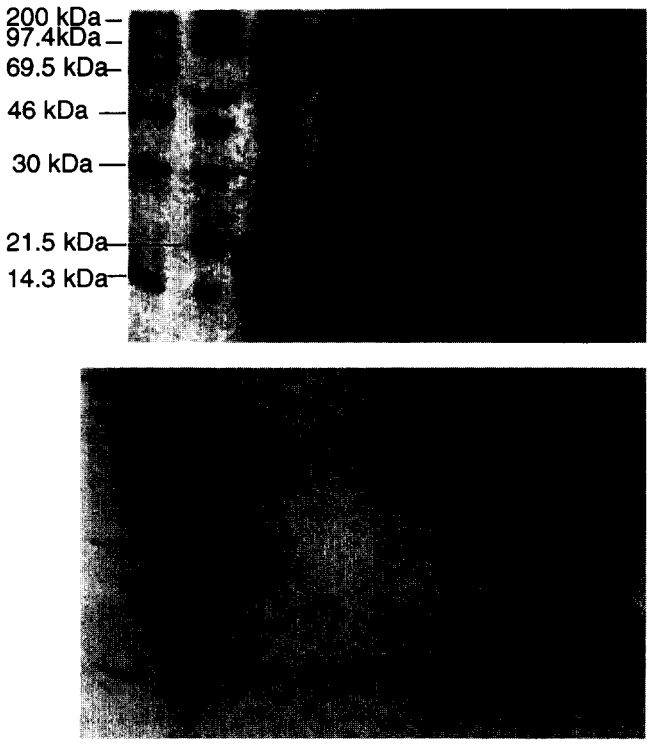

Fig. 2. Non-specific labeling of GroES by 8-N_3-ATP. 0.46 μM $GroES_7$ was incubated 30 s in 1 μM 8-N_3-ATP, 50 μM ATP, 20 mM Tris-HCl, pH 7.4, 100 mM KCl with: 5 mM $Mg(CH_3CO_2)_2$, 3 μM BSA (Sigma), or 3 μM rhodanese (Sigma), as indicated. Samples were then irradiated [11,12] and portions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; autoradiography (top) and Coomassie-blue stained (bottom).

sence of an observable thermodynamic interaction between GroES and nucleotide, reported in Fig. 1.

Could GroES donate ATP to GroEL? GroEL binds nucleotide near the equatorial plane which separates the two heptameric rings [19]. Upon binding GroES, an asymmetric structure is generated in which the apical GroEL domains move outward, with GroES located at the top of this cavity [9]. GroES is bound 60–70 Å from the ATP binding pocket. Thus, in addition to the lack of demonstrable affinity of GroES for nucleotides at physiological concentrations, there are considerable structural difficulties in imaging how GroES might deliver ATP to the nucleotide binding site of GroEL, as suggested [12]. Instead, one of the principal roles of GroES is its ability to 'quantize' the hydrolysis of ATP on GroEL, converting an entire ring of GroEL protomers as a unit to a lower affinity state for bound substrate protein, thus enhancing the efficiency of protein release [6].

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