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Affinity Chromatography of GroEL Chaperonin Based on Denatured Proteins: Role of Electrostatic Interactions in Regulation of GroEL Affinity for Protein Substrates

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Abstract—The chaperonin GroEL of the heat shock protein family from *Escherichia coli* cells can bind various polypeptides lacking rigid tertiary structure and thus prevent their nonspecific association and provide for acquisition of native conformation. In the present work we studied the interaction of GroEL with six denatured proteins (α-lactalbumin, ribonuclease A, egg lysozyme in the presence of dithiothreitol, pepsin, β-casein, and apocytochrome *c*) possessing negative or positive total charge at neutral pH values and different in hydrophobicity (affinity for a hydrophobic probe ANS). To prevent the influence of nonspecific association of non-native proteins on their interaction with GroEL and make easier the recording of the complexing, the proteins were covalently attached to BrCN-activated Sepharose. At low ionic strength (lower than 60 mM), tight binding of the negatively charged denatured proteins with GroEL (which is also negatively charged) needed relatively low concentrations (~10 mM) of bivalent cations Mg^{2+} or Ca^{2+}. At the high ionic strength (~600 mM), a tight complex was produced also in the absence of bivalent cations. In contrast, positively charged denatured proteins tightly interacted with GroEL irrespectively of the presence of bivalent cations and ionic strength of the solution (from 20 to 600 mM). These features of GroEL interaction with positively and negatively charged denatured proteins were confirmed by polarized fluorescence (fluorescence anisotropy). The findings suggest that the affinity of GroEL for denatured proteins can be determined by the balance of hydrophobic and electrostatic interactions.

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Key words: chaperonins, GroEL, affinity chromatography, protein–protein interactions

The heat shock protein GroEL (hsp60) consisting of 14 identical 57-kD subunits combined in two ring structures [1] is a component of the chaperon system of *Escherichia coli* cells [2]. One of the main functions of GroEL is the binding of various polypeptide chains lacking rigid tertiary structure, which prevents their nonspecific association and provides for further adequate folding or degradation [3-6]. Hydrophobic interactions are supposed to be a main factor stabilizing the GroEL complex with non-native proteins [7, 8]. However, electrostatic interactions have been recently shown to also play an important role in the complexing of GroEL with non-native proteins [9-11]. Potassium and magnesium ions, adenine nucleotides (ATP and ADP), and the oligomeric (seven-subunit) co-chaperonin GroES are ligands of GroEL [12-15]. The interaction of GroEL with adenine nucleotides and especially with co-chaperonin GroES decreases the affinity of the chaperonin for non-native proteins [16-21]. GroEL is also known to have a weak ATPase activity, i.e., it hydrolyzes ATP to ADP [5, 15, 16].

The interaction of GroEL with non-native proteins is usually investigated using renaturing proteins, which interact with GroEL during the early stages of folding [12, 18, 21-23]. But in this case, investigation in the presence of GroEL ligands is complicated by a rather rapid folding of the protein target to the native conformation, which prevents the interaction of the protein with GroEL. Proteins, native conformation of which is permanently disturbed under conditions native for GroEL, are more suitable for such investigations. Such proteins are exemplified by pepsin [9, 24], apocytochrome *c* [25, 26], β-casein [27, 28], and proteins with reduced intramolecular

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disulfide bonds [29, 30]. However, proteins in non-native conformations often aggregate in the absence of GroEL, or in the case of their molar excess with respect to the chaperon, and this significantly narrows the choice of protein targets suitable for studies. Aggregation of denatured protein targets can be avoided by their covalent attachment to a chromatographic carrier [31]. This markedly facilitates the recording of the complexing of GroEL with protein targets because it allows us to analyze elution profiles.

In the present work we studied the interaction of GroEL with some non-native protein targets covalently attached to Sepharose and different in their charge at neutral pH and in hydrophobicity depending on the ionic strength of solution and the presence of bivalent cations (Mg^{2+} and Ca^{2+}). We found that for the tight complexing of GroEL with negatively charged denatured proteins (α-lactalbumin in the presence of dithiothreitol (DTT), pepsin, and β-casein) low concentrations (~10 mM) of bivalent cations (Mg^{2+} and Ca^{2+}) or high ionic strength (~600 mM NaCl or KCl) is necessary. The formation of tight GroEL complexes with positively charged denatured proteins (lysozyme and ribonuclease A in the presence of DTT and also with apocytochrome *c*) does not depend on bivalent cations or ionic strength. These findings suggest that the affinity of GroEL for denatured proteins can be regulated through changing the ratio of hydrophobic and electrostatic interactions.

MATERIALS AND METHODS

Proteins. The following protein targets for GroEL from Sigma (USA) were used: horse heart cytochrome *c* (which was used to prepare apocytochrome by the method described in [26]), bovine pancreatic ribonuclease A, human milk α-lactalbumin, bovine milk β-casein, and porcine gastric mucosa pepsin; chicken egg lysozyme was from Reakhim (Russia). Pepsin, apocytochrome *c*, and β-casein are already denatured at neutral pH [24-28]. Egg lysozyme, α-lactalbumin, and ribonuclease A were denatured at neutral pH through reduction of the intramolecular disulfide bonds by addition of 20 mM DTT.

Fluorescein-labeled proteins were prepared as follows: freeze-dried proteins (~10 mg) were dissolved in 1.9 ml of 0.15 M $KNaCO_3$ (pH 9.1) and supplemented with 100 μl of 40 mM fluorescein isothiocyanate. The reaction mixture was incubated in the dark for 5 h at 20°C. Then the mixture was desalted on a PD-10 column equilibrated with 25 mM histidine (pH 5.85). The labeled protein was additionally separated from low molecular weight components, and the protein molecules with different content of the covalently attached label were separated by ion-exchange chromatography on a MonoQ HR 5/5 column in 25 mM histidine (pH 5.85) in a gradient of 0-1.0 M NaCl. The eluted peaks were dialyzed against 500 ml of 20 mM Tris-HCl (pH 7.5). The number of fluorescent labels per protein molecule was determined using the absorption coefficient of fluorescein of 87,000 $M^{-1}\cdot cm^{-1}$ at 490 nm. In subsequent work, proteins with one fluorescent label per molecule were used.

GroEL was isolated as described in [32] upon expression in *E. coli* strain HB101 cells of the multicopy plasmid pGroE4 (the complete groE operon of *E. coli* cloned in the EcoR1 site of the pACYC184 vector). Routinely isolated GroEL was additionally purified from tightly associated tryptophan-containing polypeptide admixtures using a Butyl-Toyopearl 650 chromatographic column (Tosoh, Japan) equilibrated with 25 mM Tris-HCl buffer (pH 7.5) supplemented with 1 mM EDTA, 1 mM DTT, and 10% ammonium sulfate. The ammonium sulfate concentration in the elution buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA) was decreased linearly, and GroEL was eluted from the column at zero concentration of ammonium sulfate [33]. The purity of the GroEL preparation was tested by SDS-PAGE [34] and fluorescence spectroscopy by the absence of the tryptophan fluorescence of GroEL [35]. The protein concentration was determined spectrophotometrically by absorption at 280 nm using the absorption coefficients ($A^{1\%}_{1cm}$) of 9 for apocytochrome *c*, 6 for ribonuclease A, 16 for α-lactalbumin, 27 for lysozyme, 5 for β-casein, 14 for pepsin, and 1.9 for GroEL. The molecular weight and charge of the proteins at pH 7.5 were calculated by their amino acid sequences (aspartic and glutamic acids were negatively charged, arginine and lysine were positive charged).

Reagents. To prepare affinity carriers and buffer systems, CNBr-activated Sepharose 4B from Pharmacia Biotech (Sweden), KCl, $CaCl_2$, $MgCl_2$, and urea from Reakhim, Tris, DTT, and ammonium 8-anilinonaphthalene-1-sulfonate (ANS) from Serva (Germany) were used.

Preparation of affinity carriers. Affinity carriers were prepared based on CNBr-activated Sepharose 4B as described in [36]. The protein (20 mg) was incubated in buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 9.0) with 6 ml of BrCN-activated Sepharose with constant stirring for 16 h at 4°C. Unreacted protein (always ~10%) was washed off in the same buffer. The remaining active groups were blocked by incubation in 0.1 M Tris-HCl buffer (pH 8.0) for 16 h at 4°C. The resulting affinity carrier was washed thrice (each cycle included washing in 0.1 M sodium acetate buffer, pH 4.0, supplemented with 0.5 M NaCl, and then washing in 0.1 M Tris-HCl buffer, pH 8.0, supplemented with 0.5 M NaCl). The carriers were stored at 4°C.

Affinity chromatography. Chromatographic columns (5 × 25 mm) were used. Experiments were performed at room temperature in the standard 20 mM Tris-HCl buffer (pH 7.5) supplemented with 20 mM DTT (only on using disulfide-containing target proteins: ribonuclease A, α-lactalbumin, and lysozyme), and also NaCl, $MgCl_2$, $CaCl_2$, EDTA, and ADP. The flow rate was maintained

constant at 10 ml/h with a Minipuls 3 peristaltic pump (Gilson, France). GroEL was applied onto the affinity column at the concentration of ~2 mg/ml in the volume of 150 μl. Fractions 0.5 ml in volume were dialyzed, their fluorescence was measured at 310 nm (excitation at 275 nm) with an RF-5301PC spectrophotometer (Shimadzu, Japan), ATPase activities were determined [37], and the fractions were also subjected to electrophoresis in polyacrylamide gel.

Fluorimetry. *Assessment of hydrophobicity of denatured proteins with fluorescent hydrophobic probe ANS.* To specimens of the target proteins in 20 mM Tris-HCl buffer (pH 7.5) supplemented with 30 mM KCl, 10 mM $MgCl_2$ (and also 20 mM DTT in the case of ribonuclease A, α-lactalbumin, and lysozyme), the hydrophobic probe ANS was added (to final concentrations of proteins and ANS equal to 1.4 and 28 μM, respectively). The fluorescence spectra of ANS in the absence and presence of the non-native target proteins were measured at 20°C. The excitation wavelength was 340 nm, and the fluorescence spectra were recorded in the range of 400-600 nm. The affinity of ANS to the protein target was evaluated by the difference in the ANS fluorescence at 480 nm in the presence and absence of the proteins.

Fluorescence anisotropy was measured and titration was performed in quartz cuvettes (1 × 1 × 4 cm) at 20°C. The titration was performed directly in the cuvette using a magnetic stirrer, and the measurement was performed after incubation of the specimen for 4 min. The fluorescence of the fluorescein-labeled proteins was excited at the wavelength of 490 nm, and the emission was recorded at 520 nm. Polarizers in the excitation and emission channels were manually regulated to select horizontally ($I_\perp$) and vertically (I_{II}) polarized components of the excitation and fluorescence light. The fluorescence anisotropy (A) was calculated using a standard ratio [38]:

$$A = \frac{I_{II} - I_\perp}{I_{II} + 2I_\perp} .$$

Binding of fluorescein-labeled pepsin with GroEL was analyzed by high performance gel chromatography in 20 mM Tris-HCl buffer (pH 7.5) supplemented with 30 mM KCl and containing (when indicated) $MgCl_2$, EDTA, and ADP, using a Superose 6 HR 10/30 column. Gel filtration was performed at the rate of 0.4 ml/min at 20°C. The chromatographic profile of fluorescein-labeled pepsin was recorded by fluorescence at 520 nm using an FLD-6A flow fluorimeter (Shimadzu).

RESULTS AND DISCUSSION

Physicochemical features of denatured proteins used for studies on their interaction with GroEL. Certain physicochemical features of some protein targets used in the present work were determined earlier. Thus, at neutral pH apocytochrome *c* had a low content of regular secondary structure as compared to the native state (holocytochrome *c*) and had no globular structure [25, 26]. β-Casein in solution had slightly more regular secondary structure compared to apocytochrome *c* but also had no globular structure [27, 28]. In contrast, pepsin at neutral pH, although denatured, retained a considerable part of its secondary structure and was highly compact [9, 24]. A partial or complete reduction of the intramolecular disulfide bonds in lysozyme, α-lactalbumin, and ribonuclease A at neutral pH led to denaturation of these proteins (the loss of the tertiary structure rigidity and partial destruction of the regular secondary structure) [29, 30].

In the present work, in addition to the known structural characteristics of the denatured proteins under study, it was important to get information about their "hydrophobicity", because hydrophobic interactions are principal for GroEL complexing with protein targets under physiological conditions [7, 8]. The hydrophobicity of the denatured proteins was assessed by their affinity for the hydrophobic fluorescent probe ANS often used to evaluate the degree of exposition into solution of protein hydrophobic clusters capable of binding this probe [39].

Figure 1 presents fluorescence spectra of ANS in the presence of the native and denatured proteins. Denatured α-lactalbumin and lysozyme, as well as β-casein and apocytochrome *c*, displayed higher affinity for the hydrophobic probe ANS than native lysozyme and α-lactalbumin. However, denatured pepsin and ribonuclease A weakly bound ANS (possibly, because of the low exposition of the hydrophobic clusters capable of binding ANS).

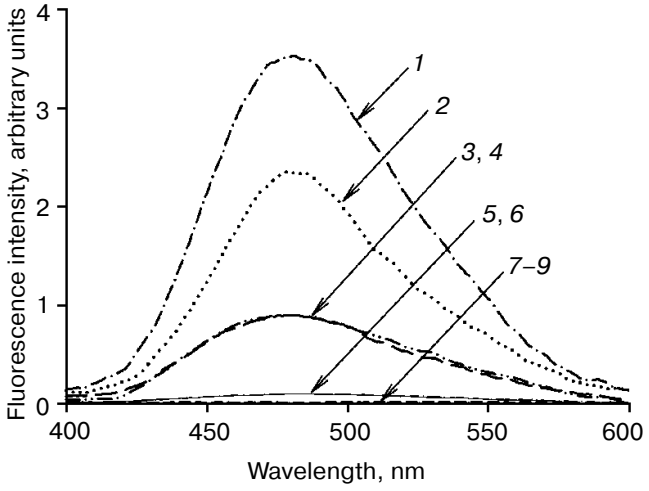

Fig. 1. Fluorescence spectra of ANS (λ_{ex} = 340 nm) in the presence of denatured α-lactalbumin (*1*), denatured lysozyme (*2*), β-casein (*3*), apocytochrome *c* (*4*), denatured pepsin (*5*), denatured ribonuclease A (*6*), and native α-lactalbumin, lysozyme, and ribonuclease A (*7-9*).

Table 1. Physicochemical features of protein substrates of GroEL

Protein	Denaturation conditions*	Charge at pH 7.5	Weight, kD	Hydrophobicity ($I^{ANS}_{480\ nm}$), arbitrary units
Apocytochrome *c*	pH 7.5	+9	11.7	0.9
Lysozyme	pH 7.5, 20 mM DTT	+8	14.3	2.3
Ribonuclease A	pH 7.5, 20 mM DTT	+4	13.7	0.1
α-Lactalbumin	pH 7.5, 20 mM DTT	–7	14.1	3.5
β-Casein	pH 7.5	–8	23.6	0.9
Pepsin	pH 7.5	–32	34.6	0.1

* The buffer contained 20 mM Tris-HCl and 30 mM KCl.

Thus, the denatured proteins used in the present work were different in size, charge at neutral pH, structure, and affinity for the hydrophobic probe ANS, or "hydrophobicity" (Table 1).

Affinity chromatography of GroEL on carriers with covalently attached denatured proteins. The attachment of the proteins to Sepharose allowed us to avoid irreversible intermolecular association usually observed in solutions of denatured proteins in moderate concentrations. Moreover, it made easier recording the GroEL complexes with the target proteins, it was sufficient to analyze profiles of the chaperonin application and elution using electrophoresis in polyacrylamide gel and measurement of the ATPase activity.

Figure 2 exemplifies the typical affinity chromatogram of GroEL on the affinity carrier with covalently attached α-lactalbumin. With the native conformation of α-lactalbumin (curve *1*), GroEL did not bind with the carrier and was removed with the free volume. But when the affinity column was equilibrated with the same buffer but containing 20 mM DTT, which reduced the intramolecular disulfide bonds of α-lactalbumin, GroEL was not detected in the free volume, i.e., it bound with the affinity carrier (curve *2*). In this case, native GroEL could be eluted from the affinity carrier with the same buffer in the presence of 20 mM Mg-ADP strongly weakening the GroEL complex with the non-native protein [17, 21, 40] (curve *2*). If the nativity of eluted GroEL was not necessary, the GroEL bound with the affinity carrier could be eluted with 6 M urea, the column washed with urea-free buffer could be used repeatedly, and the elution results could be analyzed by SDS-PAGE.

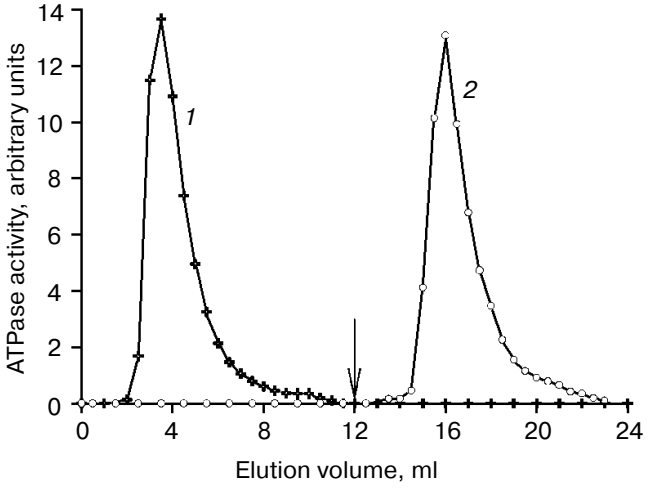

Fig. 2. Application and elution profiles of GroEL on column with affinity carrier (Sepharose with covalently attached α-lactalbumin) recorded by the ATPase activity: *1*) under conditions native for α-lactalbumin (20 mM Tris-HCl, 30 mM KCl, 10 mM $MgCl_2$, pH 7.5); *2*) under conditions of α-lactalbumin denaturation (20 mM Tris-HCl, 30 mM KCl, 10 mM $MgCl_2$, 20 mM DTT, pH 7.5). The arrow indicates the buffer change to 20 mM Tris-HCl, 20 mM Mg-ADP (pH 7.5).

To determine the role of hydrophobic and electrostatic interactions in the complexing of GroEL with non-native proteins, affinity chromatography of GroEL was performed with different carriers (Table 1) in a wide range of concentrations of bivalent cations (Mg^{2+} or Ca^{2+}) and of ionic strength (NaCl concentration) at pH 7.5. Results of these experiments are summarized in Table 2. At low ionic strength of the solution (20 mM Tris-HCl, pH 7.5) and in the absence of bivalent cations (Mg^{2+} or Ca^{2+}), GroEL did not bind with the denatured proteins negatively charged at pH 7.5 (pepsin, β-casein, and α-lactalbumin) but bound with the positively charged denatured proteins (apocytochrome *c*, ribonuclease A, and lysozyme). Among both negatively and positively charged denatured proteins there were proteins with high affinity for the hydrophobic ANS probe (high "hydrophobicity") and with low affinity (low "hydrophobicity") (Fig. 1 and Table 1). Thus, at the low ionic strength and in the absence of bivalent cations the denatured state of the negatively charged protein targets was insufficient for tight complexing with GroEL. The calculated total charge of GroEL at pH 7.5 was negative (–19 per subunit, –266 per whole oligomer). Therefore, it was suggested that the hydrophobic interaction of GroEL with a negatively charged denatured protein at low ionic strength of the

solution should be fully compensated by electrostatic repulsion of their similarly charged molecules. This seems to be supported by the tight complexing of GroEL with the negatively charged denatured proteins owing to suppression of their electrostatic repulsion on the increase in the NaCl concentration to 600 mM (Table 2). However, it must be taken into account that such an increase in the ionic strength of the solution could increase hydrophobic interactions, and this was observed indeed during reversed-phase hydrophobic chromatography of biological macromolecules [41]. In this connection, it was significant that at the low ionic strength of the solution the high affinity of GroEL for negatively charged denatured proteins was provided for by relatively low concentrations (~10 mM) of bivalent cations (Mg^{2+} or Ca^{2+}) (Table 2) [42]. Magnesium ions are known to significantly enhance the resistance of GroEL to high temperature [43] and urea [44]. It seemed that this increase in the stability was caused by Mg^{2+} binding with the sites located on the GroEL surface. This binding could increase the stability of the GroEL structure as a whole and considerably lower the local negative charge on the part of its surface, possibly in the zone of immediate contact with the denatured protein, and thus decrease the electrostatic repulsion.

Thus, the tight complexing of GroEL with the negatively charged denatured proteins significantly depended on suppression of their electrostatic repulsion, which seemed to occur under physiological conditions due to the appropriate ionic strength (~150 mM) and the presence of Mg^{2+} and Ca^{2+}. Moreover, this specific feature allowed native GroEL to be eluted from affinity carriers based on negatively charged denatured proteins using a low ionic strength buffer supplemented with EDTA (Table 2). On the other hand, the absence of bivalent ions or increasing the ionic strength to 600 mM had no influence on the GroEL complexing with the positively charged denatured proteins (Table 2). In this case, both hydrophobic and coulombic interactions seemed to be directed for enhancement of the affinity of negatively charged GroEL for the positively charged denatured pro-

Table 2. Influence of environmental conditions and ligands on the interaction of GroEL with affinity carriers based on denatured proteins

Denatured protein	Application conditions	Elution conditions	Presence of complexes
Apocytochrome *c*	A + 5 mM EDTA	A + 20 mM Mg-ADP	+
	A + 10 mM $MgCl_2$	A + 20 mM Mg-ADP	+
	A + 600 mM NaCl	A + 20 mM Mg-ADP	+
Lysozyme	B + 5 mM EDTA	B + 6 M urea	+
	B + 10 mM $MgCl_2$	B + 6 M urea	+
	B + 600 mM NaCl	B + 6 M urea	+
Ribonuclease A	B + 5 mM EDTA	B + 20 mM Mg-ADP	+
	B + 10 mM $MgCl_2$	B + 20 mM Mg-ADP	+
	B + 600 mM NaCl	B + 20 mM Mg-ADP	+
α-Lactalbumin	B + 5 mM EDTA	– (GroEL in the free volume)	–
	B + 10 mM ($MgCl_2$ or $CaCl_2$)	B + 5 mM EDTA	+
	B + 5 mM EDTA, 600 mM NaCl	B + 5 mM EDTA	+
β-Casein	A + 5 mM EDTA	– (GroEL in the free volume)	–
	A + 10 mM ($MgCl_2$ or $CaCl_2$)	A + 5 mM EDTA	+
	A + 5 mM EDTA, 600 mM NaCl	A + 5 mM EDTA	+
Pepsin	A + 5 mM EDTA	– (GroEL in the free volume)	–
	A + 10 mM ($MgCl_2$ or $CaCl_2$)	A + 5 mM EDTA	+
	A + 5 mM EDTA, 600 mM NaCl	A + 5 mM EDTA	+

Note: A) 20 mM Tris-HCl (pH 7.5); B) 20 mM Tris-HCl, 20 mM DTT (pH 7.5).

tein. In some cases, the interaction of GroEL with the positively charged denatured proteins could be weakened in the presence of adenine nucleotides (e.g., Mg-ADP (Table 2)). However, the complexing of GroEL with positively charged and strongly hydrophobic lysozyme denatured in the presence of DTT (Table 1) was virtually not weakened in the presence of Mg-ADP (see also [20]). In this case, the complex could be effectively dissociated, in particular by elution with 6 M urea (Table 2).

Interaction of GroEL with denatured proteins in solution. To check the possible influence of the chromatographic carrier (Sepharose) and covalent attachment of the proteins to it on the results obtained by affinity chromatography of GroEL, the interactions of GroEL with fluorescein-labeled denatured pepsin, α-lactalbumin, and lysozyme were studied at pH 7.5 using high performance gel chromatography and fluorescence anisotropy. Figure 3 presents the elution profiles of fluorescein-labeled pepsin under different conditions. Fluorescein-labeled pepsin denatured at pH 7.5 was eluted as a mixture of monomers and dimers and in the absence of Mg^{2+} did not interact with GroEL even in the presence of 30 mM KCl. The addition into the buffer system of 10 mM $MgCl_2$ resulted in elution of denatured pepsin together with GroEL (i.e., tight complexing of pepsin with GroEL). The addition into the buffer system of 1 mM ADP strongly destabilized the GroEL complex with denatured pepsin.

The effect of an increase in the bivalent ion concentration and ionic strength on the GroEL affinity for the negatively and positively charged denatured proteins in

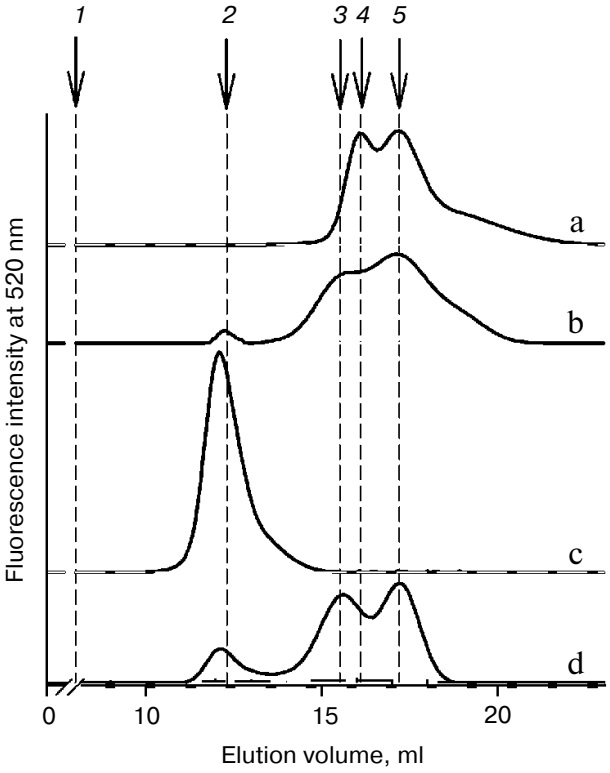

Fig. 3. Gel filtration profiles of fluorescein-labeled denatured pepsin (pH 7.5) in the absence and presence of GroEL (molar ratio 1 : 1) and its ligands: a) pepsin; b) pepsin + GroEL; c) pepsin + GroEL + 10 mM $MgCl_2$; d) pepsin + GroEL + 10 mM $MgCl_2$ + 1 mM Mg-ADP. The figures above the arrows indicate elution volumes: *1*) free volume; *2*) GroEL; *3*, *4*, *5*) trimers, dimers, and monomers of denatured pepsin, respectively. Gel filtration was performed in the standard buffer: 20 mM Tris-HCl, 30 mM KCl (pH 7.5).

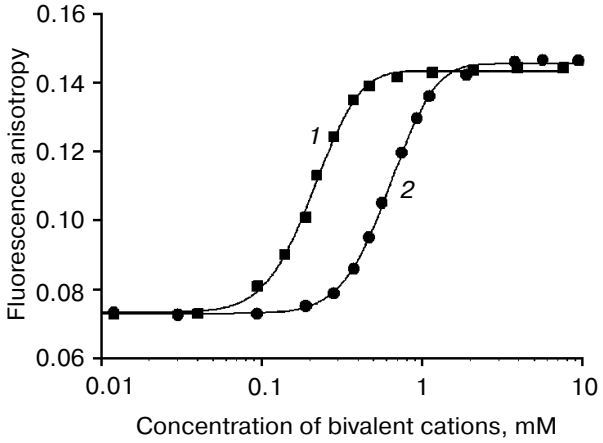

Fig. 4. Dependence of fluorescence anisotropy of fluorescein-labeled denatured α-lactalbumin (protein concentration 10^{-7} M) in the presence of GroEL (at ratio 1 : 1) on the concentration of Ca^{2+} (curve *1*) and Mg^{2+} (curve *2*). The ion concentrations at the half-transition points were 0.21 and 0.62 mM for Ca^{2+} and Mg^{2+}, respectively. Buffer: 20 mM Tris-HCl, 30 mM KCl, 20 mM DTT (pH 7.5).

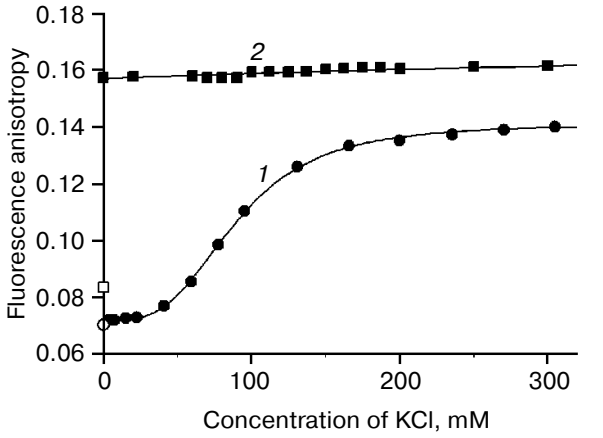

Fig. 5. Dependence of fluorescence anisotropy of fluorescein-labeled denatured α-lactalbumin (*1*) and lysozyme (*2*) in the presence of GroEL (at ratio 1 : 1) on the KCl concentration. The protein concentrations were 10^{-7} M. Buffer: 20 mM Tris-HCl, 20 mM DTT (pH 7.5). Open symbols indicate the fluorescence anisotropy of the corresponding proteins in the absence of GroEL.

solution was also studied by polarized fluorescence (fluorescence anisotropy). Figures 4 and 5 present, respectively, the titration curves with bivalent (Mg^{2+} and Ca^{2+}) and monovalent (K^+) cations of equimolar mixtures of GroEL with the negatively and positively charged denatured proteins. At the low ionic strength of the solution, increase in the bivalent ion concentration stabilized the GroEL complex with negatively charged denatured α-lactalbumin (Fig. 4), and Ca^{2+} was more effective than Mg^{2+}. The increase in the ionic strength in the absence of bivalent cations also resulted in stabilization of the complex of negatively charged α-lactalbumin with GroEL (Fig. 5). Nevertheless, the increase in the ionic strength (to 600 mM NaCl) did not influence the complexing of GroEL with positively charged denatured lysozyme (Fig. 5).

Thus, specific features of the interaction of GroEL with negatively and positively charged denatured proteins covalently attached to Sepharose were the same as in solution (compare the data presented in Table 2 and Figs. 3-5). We earlier showed also that denatured lysozyme (with reduced disulfide bonds) tightly complexed with GroEL in solution, and this prevented its intermolecular association. However, the presence of Mg-ADP only slightly influenced the tightness of this complex [20]. Taking all the abovementioned into account, it is concluded that the protein attachment to Sepharose does not significantly affect the physicochemical properties of the denatured proteins and specific features of their interaction with GroEL.

Note in conclusion that affinity chromatography based on denatured proteins can be used not only to study functions of GroEL and other chaperons. We have earlier shown [35] that such an approach can be effectively used for the virtually one-stage purification of GroEL in preparative amounts. Because other chaperonins of both prokaryotic and eukaryotic cells interact with polypeptides lacking rigid tertiary structure, affinity chromatography based on denatured proteins is also promising for their efficient isolation.

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