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Ultracentrifugal analysis of the quaternary structure of the *raf* repressor from *Escherichia coli*

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The *raf* repressor from *Escherichia coli* regulates the expression of the plasmid-borne *raf* operon by switching between active and inactive conformational states. Ultracentrifugal analysis of the largely purified repressor proves the DNA-free protein to undergo concentration-dependent dissociation-association. High-speed sedimentation equilibria show that the 72 kDa dimer prevails under méniscus depletion conditions. At intracellular concentrations the 144 kDa dimer-of-dimers is the dominating species. It is suggested that the tetrameric structure of the *raf* repressor is involved in the recognition of the 18-basepair operator DNA.

Analytical ultracentrifugation; Dissociation-association; Quaternary structure; Repressor, *raf*; Sedimentation analysis

1. INTRODUCTION

The *raf* repressor has been shown to regulate expression of the plasmid-borne *raf* operon, which encodes functions required for the uptake and hydrolysis of raffinose in *Escherichia coli* [1,2]. The repressor gene, *rafR*, has been sequenced, the deduced protein monomer M_r being 36700 [2]. Overexpression of *rafR* resulted in inclusion bodies, from which active repressor was recovered by solubilization in 0.1% sodium dodecyl sulfate and subsequent dilution in buffer [3]. Gel-retardation analysis [4] revealed the specific binding of *raf* repressor to 18-bp palindromic raf operator DNA sequences preceding the structural genes. Molibiose, a specific inducer of the *raf* operon, released the repressor-operator complexes. This suggested an alternation of the repressor molecules between active and inactive conformational states, a property only exhibited by oligomeric proteins [5].

The quaternary structure of other known repressor molecules is either dimeric or tetrameric [6,7]. To determine the yet unknown quaternary structure of the *raf* repressor, a largely purified DNA-free preparation (capable of specifically binding to raf oprator DNA) was analyzed by analytical ultracentrifugation. The results reported here indicate a concentration-dependent monomer-dimer-tetramer equilibrium with the tetramer (formed at typical intracellular concentrations) as the putative active configuration.

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2. MATERIALS AND METHODS

Assay of *raf* repressor activity employed the gel-retardation procedure [4] using 3'labelled DNA fragments that contained either two or four palindromic *raf* operators for repressor binding, as has been described [3]. Protein concentrations during purification were determined according to Lowry et al. [8], using bovine serum albumin as a standard. The concentration of purified repressor was determined spectrophotometrically using an extinction coefficient $A^{0.1\%}_{280\,nm} = 1.5$. The isolation of active *raf* repressor from purified inclusion bodies was performed as in [3]. For analytical ultracentrifugation, dialyzed repressor protein was diluted to 400 μg/ml in binding buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM EDTA) that contained different concentrations of dithiothreitol (0, 2, 4, 10 mM) or mercaptoethanol (100 mM).

Sedimentation experiments were performed in a Beckman Spinco model E analytical ultracentrifuge equipped with a high intensity light source and a UV scanning system. Double sector cells (12 mm pathlength) with sapphire windows were used in an AnG rotor. To detect possible concentration-dependent dissociation, heterogeneity and non-ideality, the meniscus depletion technique was applied over a wide range of protein concentrations (5 μg/ml $-$2 mg/ml), fill-heights and rotor speeds (8000–20000 rpm); scanning wavelengths were 230 and 280 nm, respectively. The temperature in all ultracentrifuge experiments was 2 and 20 ± 1°C, respectively. Sedimentation coefficients were determined at 44000 rpm, plotting ln r vs t, and correcting for 20°C and water viscosity. Sedimentation equilibrium experiments with rotor speeds 16000, 12000 and 8000 rpm were evaluated from ln c vs r^2 plots making use of a computer program developed by G. Böhm, Regensburg. The partial specific volume was calculated from the amino acid composition [10].

3. RESULTS AND DISCUSSION

The molecular mass of the *raf* repressor from *Escherichia coli* differs, depending on the concentration of the protein and the amount of sodium dodecyl sulfate used as solubilizing agent. At 0.01% high

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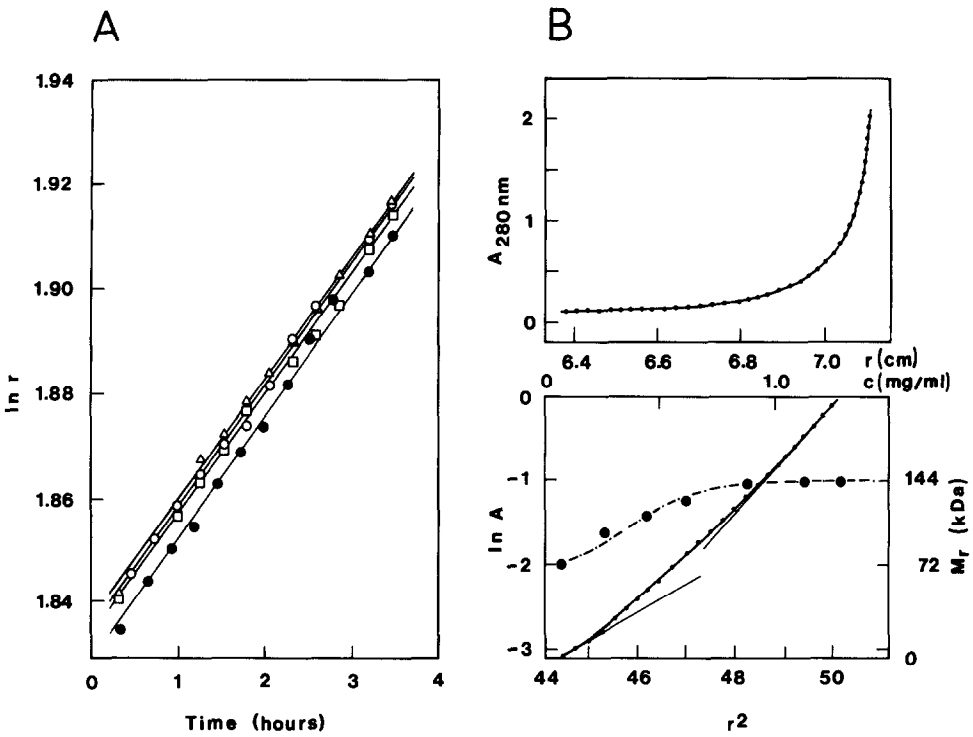

Fig.1. Sedimentation velocity and sedimentation equilibrium of *raf* repressor. Experiments were performed at 2°C in 10 mM Tris-HCl buffer, pH 8.0, plus 10 mM KCl and 1 mM EDTA. (A) Semilogarithmic plot (ln r vs t) of sedimentation velocity runs at 44000 rpm. Scanning wavelength 280 nm. Protein concentration 0.4 mg/ml, in the absence of dithiothreitol (○), and in the presence of 2 mM (△), 4 mM (□) dithiothreitol, and 100 mM β-mercaptoethanol (●), respectively. (B) Tetramer-dimer dissociation equilibrium calculated for high-speed sedimentation equilibrium runs at 12000 and 8000 rpm. Data refer to experiments with different filling heights and scanning wavelengths. Initial protein concentration 0.4 mg/ml in the presence of 4 mM dithiothreitol. (Upper frame) Equilibrium at 12000 rpm, monitored at 280 nm. (Lower frame) Left-hand ordinate and lower abscissa, ln A vs r^2 linearization; righthand ordinate and upper abscissa (●), concentration dependence of the molecular mass.

molecular weight aggregates prevail: about 90% of the protein are present as high molecular weight aggregates, which sediment to the bottom of the ultracentrifuge cell during acceleration (≤20000 rpm). The remaining 10% migrate as a single species with a sedimentation coefficient $s_{20,w} = 3.5 \pm 0.1 \times 10^{-13}$ s. At concentrations below 0.05 mg/ml) (monitored at 230 nm), the molecular mass is found to extrapolate to 36000 ± 3400 Da, corresponding to the calculated molecular weight of the polypeptide chain (correlation coefficient 0.9994).

After equilibrium dialysis of the 0.01% sodium dodecyl sulfate solution of the protein against excess buffer (residual sodium dodecyl sulfate concentration ≤0.001%), aggregation does not exceed 10%. The major fraction of the protein forms a well-defined sedimentation boundary. The sedimentation coefficient at $c = 0.5$ mg/ml is $s_{20,w} = 6.1 \pm 0.2 \times 10^{-13}$ s, independent of the amount of reducing agent present (fig.1A). Obviously, the requirement of dithiothreitol for specific binding to operator DNA sequences [3] has no effect on

Table 1

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Conditions[a]				12000 rpm		8000 rpm	
[SDS] (%)	[DTT] (mM)	T (°C)	$s_{20,w}$ (S)	M_{min} (kDa)	M_{max} (kDa)	M_{min} (kDa)	M_{max} (kDa)
0.01	0	24	3.54 ± 0.11	33.7	aggr.		
0.001	0	2	6.1 ± 0.1	80	136 ± 10	135	160 ± 10
0.001	2	2	6.1 ± 0.1	116	142 ± 6	129	146 ± 6
0.001	4	2	6.0 ± 0.1	106	149 ± 8	126	157 ± 8
0.001	100[b]	2	6.1 ± 0.1	108	146 ± 6	130	175 ± 10

[a] [SDS], residual sodium dodecyl sulfate concentration; [DTT], dithiothreitol concentration; T, temperature

[b] β-Mercaptoethanol concentration

the quaternary structure of the protein. Assuming the hydrodynamic properties typical for globular proteins to be valid for the repressor molecule, the observed sedimentation coefficient allows a molecular mass of 130–150 kDa to be predicted. Fig.1B and table 1 show that this value is confirmed by high-speed sedimentation equilibrium experiments using the meniscus depletion technique in a concentration range between 0.05 and 2 mg/ml. Thus, we may conclude that at sodium dodecyl sulfate concentrations below 0.001% the solubilized *raf* repressor in the absence of its target DNA is a tetramer. Homogeneity and stability of the protein are corroborated by the occurence of a single sedimentation boundary and identical standard M_r moments at varying angular velocity. Only at high dilution (≤ 0.05 mg/ml) and high rotor speed, dissociation to dimers becomes detectable: a significant curvature in the ln c vs r^2 diagrams is observed with limiting weight average molecular masses of the order of 80 ± 8 kDa and 145.2 ± 2.5 kDa, respectively (fig.1B). Under the conditions of the biological binding assay ($\leq 0.001\%$ sodium dodecyl sulfate, 0.05 mg/ml protein concentration), the tetrameter is the predominant species. In the bottom region of the ultracentrifuge cell, and at low rotor speed (8000 rpm), the weight average molecular mass slightly exceeds the tetramer. However, even after long-term equilibration at room temperature, aggregation is below 10%.

4. CONCLUSIONS

Raf repressor exhibits concentration-dependent dissociation-association. The 36 kDa monomer is observed only in the presence of excess solubilizing agent. Under this condition, most of the protein forms aggregates. Obviously. the subunit interface of the dimer is strongly hydrophobic. At sodium dodecyl sulfate concentrations below 0.001%, the dimer is the final dissociation product, even at exceedingly low protein concentration. At typical cellular concentrations, the tetramer is the dominating species; it represents the stable state of association even at concentrations in the mg range. As in the case of other DNA binding proteins, pairwise binding may be assumed to play a role in the binding of the repressor to its operator DNA. Whereas most functional repressors studied assume dimeric conformations, the well-known *lac* repressor from *E. coli* is operative as a tetramer [6,7], a notion in line with our analysis of the *raf* repressor. The functional significance of this conformation is not known, since size and symmetry considerations and the detailed structure analyses of other analogous repressor-operator systems [6,7] suggest that the helix-turn-helix motifs of only two protein subunits interact with the 18-bp palindromic *raf* operator DNA. The assumption that the two adjacent *raf* operators (separated by only 3 bp) are bound simultaneously by two surfaces of the tetrameric repressor were disproved by in vitro binding studies which demonstrated sequential binding of individual *raf* repressor molecules [3]. Two naturally separated binding sites in the *lac* system (the second located in the *lac* coding region) have been recently implicated in the cooperative repression of the *lac* promotor [11]; moreover, in vitro experiments with artificial constructs containing two operator sites at varying distances have demonstrated the formation of a DNA loop by binding one *lac* repressor tetramer to the two *lac* operators [12]. Since presently no structural information is available, no details with respect to the topology of the protein-DNA complex and the specific protein-DNA interactions can be given. However, the present data indicate that the dimer-of-dimers struture of the *raf* repressor serves as the preferred configuration interacting with the operator DNA.

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