PREFERENTIAL INTERACTION PARAMETERS FOR F_1-ATPase FROM A THERMOPHILIC BACTERIUM (PS3)

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Received 16 November 1981

1. Introduction

The proton translocating ATPase of energy transducing membranes couples the vectorial transport of protons through the membrane to synthesize ATP. The enzyme can be divided into an integral membrane portion, F_o, and a peripheral portion, called the coupling factor or F_1-ATPase, which contains the catalytic site of ATP synthesis and hydrolysis [1–3]. The F_1 portion of the H^+-ATPase consists of 5 different subunit species, $\alpha,\beta,\gamma,\delta$ and ϵ. The subunits have been isolated of F_1 from *Escherichia coli*, PS3 and CF_1 [4–6], and their amino acid composition and M_r-values determined [7]. The subunit stoichiometry is still controversial because of incomplete reassembly to a functional F_1 of the isolated subunits [7]. Also, the reported M_r-values of F_1 vary from 350 000–380 000, e.g., for ECF_1 [9,10], due to differences in analytical methods and preparations. However, analytical ultracentrifugation data were not treated as a multicomponent system with c_2 the mass of the protein (F_1), c_3 the added solvent component, e.g., methanol or glycerol, and c_1 the principal solvent (buffer) with c_i in g/ml solution.

This paper reports on studies of TF_1 in 0.01 M Tris–HCl buffer at pH 7.0–8.0 in the presence and absence of methanol and glycerol by means of light scattering and differential refractometry experiments, in order to detect any interactions of component three with the protein. Since these organic solvents are often used in stabilizing F_1 structures from bacterial strains, it is a prerequisite to measure the preferential interaction parameter in this multicomponent system in order to determine accurate values of the M_r-values of F_1 and to elucidate the thermodynamic stabilization of these organic co-solvents.

Abbreviations: M_r, relative molecular mass; CF_1, coupling factor from chloroplast; ECF_1, coupling factor from *E. coli*; TF_1, coupling factor from the thermophilic bacterium PS3; DTT, dithiothreitol; EDTA, ethylendiamine tetra-acetic acid, disodium salt; $\alpha,\beta,\gamma,\delta,\epsilon$, subunits in order of decreasing M_r

2. Materials and methods

F_1 from PS3 was prepared as in [5]. Protein concentrations were determined spectrophotometrically using an absorptivity value of $A_{278}^{0.1\%} = 0.79$ ml/(cm.g). The same value of the F_1-extinction coefficient was used at all solvent compositions since variation of solvent composition from water to 40% methanol or glycerol does not seriously affect the position of the absorption maximum nor the extinction coefficient between pH 7.0–8.0 in 0.01 M Tris–PO_4, containing 1 mM DTT and 0.5 mM EDTA.

The refractive index increments were measured on a photoelectrical differential refractometer at 435 nm and 20 ± 0.1°C [10]. To measure the refractive index increment at identical chemical potentials of solvent components in the TF_1 solutions and the reference solvent, $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$, the protein solution was first brought to dialysis equilibrium with the solvent. The dialyzed TF_1 and the dialysates were then introduced into the 2 compartments of the differential cell for measurements. The refractive increments of methanol and/or ethanol in aqueous solutions, $(\partial n/\partial c_3)_{T,p,m_2}$, were obtained by measuring the refractive index at several solvent compositions with a Brice Phoenix precision refractometer at 435 nm and 20°C, and by drawing tangents to the plots of the refractive indexes *vs* concentration of organic solvent.

Published by Elsevier/North-Holland Biomedical Press
00145793/82/0000–0000/$02.75

Table 1

Preferential interaction of TF_1 with solvent components in the water–methanol, water–glycerol system at pH 7.8, ionic strength = 0.05 M (20°C)

% solvent (ml/ml solution)	$\left(\frac{\partial n}{\partial c_2}\right)_{T,\mu_1,\mu_3}$ (ml/g)	$\left(\frac{\partial n}{\partial c_2}\right)_{T,P,m_3}$ (ml/g)	$\left(\frac{\partial n}{\partial c_3}\right)_{T,m_2,P}$ (ml/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial \mu_3}{\partial m_2}\right)^{a}_{T,P,m_3}$ [cal/(mol in 1000 g H_2O)²]
Glycerol						
15	0.179 ± 0.002	0.185 ± 0.003	0.135	−0.039 ± 0.045	0.210 ± 0.099	1200
25	0.170 ± 0.003	0.178 ± 0.002	0.130	−0.125 ± 0.041	0.260 ± 0.098	4100
30	0.150 ± 0.002	0.171 ± 0.002	0.125	−0.474 ± 0.040	0.490 ± 0.101	6200
40	0.131 ± 0.002	0.160 ± 0.001	0.117	−0.591 ± 0.039	0.821 ± 0.081	7300
50	0.111 ± 0.002	0.151 ± 0.001	0.110	−0.940 ± 0.035	0.910 ± 0.061	9100
Methanol						
15	0.170 ± 0.002	0.175 ± 0.003	0.136	−0.045 ± 0.031	0.199 ± 0.106	1300
25	0.150 ± 0.002	0.170 ± 0.002	0.130	−0.125 ± 0.031	0.219 ± 0.100	4300
30	0.128 ± 0.003	0.168 ± 0.002	0.128	−0.213 ± 0.040	0.556 ± 0.071	6900
40	0.125 ± 0.003	0.160 ± 0.002	0.119	−0.475 ± 0.032	0.815 ± 0.061	7400
50	0.103 ± 0.002	0.652 ± 0.001	0.115	−0.951 ± 0.053	0.921 ± 0.050	9100

[a] The change in the chemical potential of glycerol or methanol induced by introduction of TF_1 into the solvent is calculated according to:

$$(\partial\mu_3/\partial m_3)_{T,P,m_2} = (RT/m_3) + R \cdot T(\partial \ln j_3/\partial m_3)_{T,P,m_2}$$

with j_3 the activity coefficient of glycerol or methanol in variation with concentration (c_3)

The equipment and procedure for light scattering measurements, as well as the data processing are essentially the same as in [11–13]. A 6–8° annulus was used to isolate the scattered light after it was shown that the scattering intensities for the protein solution were independent of angle over the range of 2–8°.

For dialysis experiments, 10 solutions of TF_1 at different concentrations were prepared in the given buffer (pH 7.0–8.0) methanol or glycerol mixture, dialyzed for 7 days against a large excess of the same solvent, and then passed through the sintered glass filter after centrifugation. Measurements were carried out then, using the dialysate as a blank.

When the scattering intensities of TF_1 solutions are measured in a water–non-aqueous solvent mixture keeping the molality of the non-aqueous solvent identical in the solvent and in the solution, the multicomponent theory [14] results in the equations:

$$H\left(\frac{\partial n}{\partial c_2}\right)^2_{T,p,m_3} \frac{c_2}{\Delta\tau} = \frac{1}{(1+D)^2}\left(\frac{1}{M_2} + 2B^\circ c_2\right) \quad (1)$$

with $H = \dfrac{32\pi^3 n^2}{3N_A\lambda^4}$

$$D = \frac{(\partial n/\partial m_3)_{T,p,m_2}}{(\partial n/\partial m_2)_{T,p,m_3}}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,p,\mu_3} \quad (2)$$

and B° is an apparent second virial coefficient [15] involving interaction constants between solute and solvent and the preferential interaction parameter, $(\partial m_3/\partial m_2)_{T,p,\mu_3}$; μ_i is the chemical potential of component i, and $\partial n_i/\partial m_i$ are the refractive index increments of component i. A plot of $H(c_2/\Delta\tau)$ as a function of concentration does not extrapolate to the true M_r of the macromolecule, but to the product of the M_r and a function of preferential interaction with solvent components. The deviation of this extrapolation, $1/(1+D^2)M_2$, from the reciprocal of the true M_r-value, $1/M_2$, is a measure of the extent of this interaction. In these equations, $\Delta\tau$ is the difference in the turbidity between the solution and that of the buffer.

3. Results

The results of differential refractometry experiments for TF_1 for the water–methanol or water–glycerol system as a function of solvent composition are listed in table 1. The corresponding values for preferential hydration according to:

$$\left(\frac{\partial\delta_3}{\partial\delta_2}\right)_{T,\mu_1,\mu_3} = \frac{M_2}{M_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} \quad (3)$$

with δ_i the concentration of component i in grams of i/gram of component 1 (water), $i = 2$ for the protein, and $i = 3$ for the added component (ethanol, methanol or glycerol); m_i is the molal concentration of component i, and M_i the M_r-value of component i. The results for TF_1 are shown in fig.1. It can be seen that for TF_1 the interaction is strongly marked by preferential hydration of the protein. The extent of this interaction increases for TF_1 over 15–25% (v/v) methanol (glycerol) and reaches 0.95 g H_2O/g protein at pH 7.8 (20°C), whereas 0.75 g H_2O/g protein is obtained when ethanol is used. The corresponding value for the same experiments conducted by adding glycerol up to 35% (v/v) is found to be 0.97 g H_2O/g protein for TF_1 and is in qualitative agreement with 0.89 g H_2O/g protein for ECF_1, obtained by means of small angle X-ray scattering experiments and density measurements [16]. However, more experiments must be conducted to establish the real thermodynamic stan-

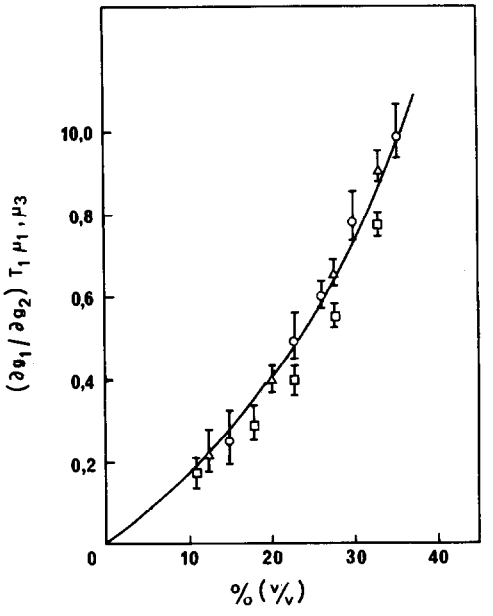

Fig.1. Preferential interaction of TF_1 with solvent components (ethanol ○, methanol △, and glycerol □) in 0.01 M Tris–PO_4, (pH 7.8) containing 1 mM DTT and 0.5 mM EDTA, 20°C.

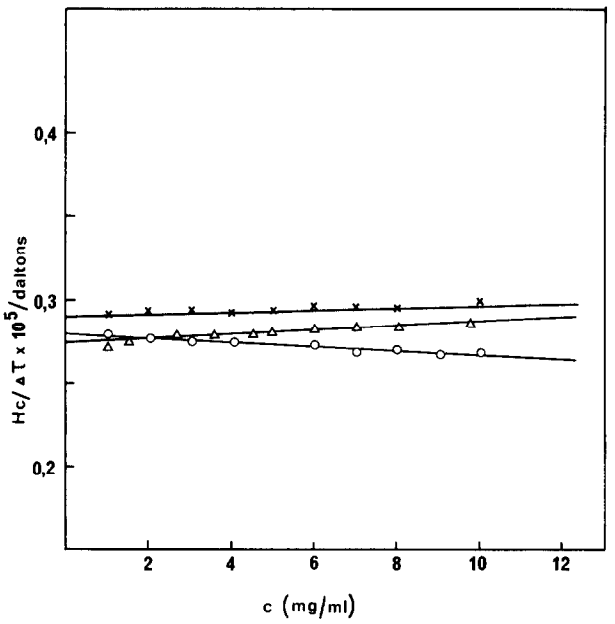

Fig.2. Light scattering of TF_1 in 0.01 M Tris–PO_4 (pH 7.8) buffer: methanol (△——△) and glycerol (○——○), at 20°C. (×——×) TF_1 in 0.01 M Tris–PO_4 (pH 7.8) containing 1 mM DTT and 0.5 mM EDTA. All at constant μ_3.

dard state of these experiments since they were performed in the absence of organic solvent. Fig.2 shows plots of $H(c_2/\Delta\tau)$ *vs* concentration of TF_1 in the buffer systems water–methanol and water–glycerol at pH 7.8, 20°C, under conditions of constant μ_3 and m_3. By dialysis measurements we found that the wt av. M_r for TF_1 is 390 000 at 25% (v/v) ethanol or methanol. The value in the presence of 30% (v/v) glycerol is 389 000 and at 40% (v/v) glycerol it is 391 000. This indicates that some aggregation is occurring (table 2). At all solvent compositions, introducing TF_1 into the water–glycerol or water–methanol system, causes the chemical potential of the organic alcohols to become more positive. This indicates repulsion between TF_1 and glycerol or methanol and, hence, a thermodynamic destabilization of the system which increases with increasing glycerol or methanol concentration. Comparison of the values of preferential hydration obtained by light scattering according to eq. (1) and (2) with those of refractometry experiments reveals that the 2 are in agreement, with an uncertainty of 3% in D due to aggregation of TF_1.

4. Discussion

These results clearly show two important features:
(1) Adding ethanol, methanol or glycerol up to 30–35% to solutions of TF_1 results in a thermodynamic destabilization of the solvent system which is manifested as an increase in the chemical potential of the organic solvent and a strong preferential hydration of the coupling factor;
(2) The aggregation of TF_1 is enhanced. In our case, the preferential interaction for TF_1 is negative which indicates preferential exclusion of the alcohols from

Table 2
Light scattering results of TF_1 in water–methanol or water–glycerol (pH 7.8)

% solvent (vol. %)	$\bar{M}_{app.2} \times 10^5$	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)
Glycerol			
25 (μ_3 = const.)	3.89 ± 0.21		
25 (m_3 = const.)	3.75 ± 0.15	−0.18 ± 0.05	0.63 ± 0.20
30 (μ_3 = const.)	3.95 ± 0.20		
30 (m_3 = const.)	3.85 ± 0.16	−1.15 ± 0.20	0.95 ± 0.25
0	3.68 ± 0.15		
Methanol			
25 (μ_3 = const.)	3.85 ± 0.25	−0.21 ± 0.05	0.71 ± 0.20
25 (m_3 = const.)	3.70 ± 0.21		
30 (μ_3 = const.)	3.91 ± 0.19	−1.30 ± 0.15	1.01 ± 0.25
30 (m_3 = const.)	3.81 ± 0.25		
0	3.65 ± 0.14		

contact with the enzyme, since $(\partial\delta_3/\partial\delta_2)_{T,\mu_1,\mu_3}$ is negative. Therefore, it acts uniformly over the entire external surface of the protein. CD and UV spectroscopic data indicate that the enzyme conformation is the same in dilute buffer and in the water–primary alcohol. Therefore, the surface area can be regarded as approximately the same in the 2 solvent systems. Moreover, aggregation causes the removal of part of this external surface area from contact with solvent by formation of TF_1–TF_1 contact reducing the absolute value of $(\partial\delta_3/\partial\delta_2)_{T,\mu_1,\mu_2}$ per monomer TF_1, the enzyme aggregates. Taking the results from table 2 and assuming that glycerol and methanol or ethanol is totally excluded from the domain of the enzyme, the minimum interaction with water is represented by the values of $(\partial\delta_i/\partial\delta_2)_{T,\mu_1,\mu_3}$ in g/g.

Thus, in 35% (v/v) glycerol, there is an effective layer of water of ~1 g H_2O/g enzyme which is impenetrable to glycerol, whereas for 40% (v/v) methanol or glycerol it is almost 1.2 g H_2O/g enzyme. This is about twice the water content determined from small angle X-ray scattering measurements [13], NMR data [17], as well as from crystallization studies of single crystals of F_1 [17–20], and 3-times the value of the normal hydrodynamic hydration of proteins [21]. It seems, therefore, that close to the external surface of the TF_1 molecule there has to be a region in which solvent composition is perturbed. Evidently, at these compositions of the solvent for TF_1, water is preferred over methanol, ethanol or glycerol, and the high value of ∂n, compared to that usually found for proteins in solvents containing inert-type solutes, suggests that water, but not glycerol or sucrose, can penetrate the interior space of TF_1.

As already suspected [22], these light scattering and differential refractometry measurements establish that the third component, e.g., methanol, ethanol or glycerol, induces aggregation of TF_1, ECF_1 and, even more strongly CF_1 so that their determined wt av. M_r-values are ~10–12% too high. Densitometry experiments for evaluating the density increments and partial specific volumes for TF_1 are consistent with the data presented and corroborate the fact that addition of alcohols significantly influences the M_r-values due to aggregation and inaccurate partial specific volumes. According to this investigation, the minimum M_r for TF_1 is 365 000 ± 10 000, similar to F_1 from *Bacillus stearothermophilus* [23] and *Escherichia coli* [10]. This value is further substantiated from M_r measurements of the reconstituted 3-subunit enzyme [23] and crystallization experiments of single crystals of SF_1 [24]. The subunit stoichiometry of the main subunits (α,β,γ) ought to be of the $\alpha_3\beta_3$ type for TF_1, ECF_1 and SF_1.

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