STABILITY AND FLEXIBILITY OF THE α-SUBUNIT OF F_1-ATPase FROM THE THERMOPHILIC BACTERIUM PS3

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1. Introduction

Proton-translocating ATPase catalyzes the terminal step of the synthesis of ATP in oxidative and photophosphorylation [1–3]. The peripheral, soluble fraction of the ATPase consists of 5 subunits: α,β,γ,δ and ε, in decreasing order of molecular weight [3]. In [4] stable subunits of TF_1-ATPase were purified from the thermophilic bacterium PS3 and TF_1 reconstituted from the purified subunits. The availability of such subunits opens the opportunity to study their physical properties, ligand binding capabilities and interaction with the other subunits.

Purified α and β subunits both have nucleotide binding sites [5,6] and it was claimed that the α-subunit has an allosteric site, while the β-subunit of TF_1 has a catalytic one [5,6]. This paper continues the studies of the conformation of the α-subunit in solution in the presence and absence of ATP by means of fluorescence experiments which indicate that the α-subunit of TF_1 is composed of a domain smaller than that of an M_r 60 000 protein. The relaxation time of the native α-subunit, as determined by fluorescence depolarization measurements, indicates a flexible structure. However, upon binding of ATP it becomes more rigid.

2. Materials and methods

The α-subunit of TF_1 from the thermophilic bacterium PS3 was prepared as in [7,8]. ATP and ADP sodium salt was purchased from Sigma (Munich).

Abbreviations: α,β,γ,δ,ε, subunits of F_1-ATPase in order of decreasing M_r; DnS, dimethylamino naphthalene-1-sulfonyl

DnS groups were conjugated to α by dansylation in 0.1 M sodium bicarbonate, (pH 8.3) at 25°C, with dimethylamino naphthalene-1-sulfonyl chloride. A dye/protein weight ratio of ~1.3–1.5% was used, and the labelled α-subunit was separated from hydrolyzed, free dye by passing through a BioGel P-10 column. The buffer used for studying α contained 50 mM K_2HPO_4 (pH 7.8), 50 mM NaCl and 0.1 mM EDTA. ATP was added at a final concentration of 50 μM to the DnS-labelled protein.

Light scattering measurements [9] and column chromatography experiments [10] indicated that the α-subunit before and after fluorescence measurements was in the monomeric state. Fluorescence measurements were made in a modified fluorimeter from Applied Photophysics (SP4, London) with automated polarization accessory and thermostatted cell holder. Polarization of fluorescence measurements were made with this instrument, also. Measurements of DnS polarization were made using polarized excitation at 340 nm and emission at 500 nm. The polarization was calculated using the formula:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

as described in [11], with $G = I_{HV}/I_{HH}$, I is the fluorescence intensity, and the first and second subscripts refer to the plane of polarization of the excitation and emission beams, respectively, with V = vertical and H = horizontal.

The relaxation time (ρ_H) was computed from the dependence of polarization on temperature, according to the Perrin equation:

$$(1/p - 1/3) = (1/p_o - 1/3)(1 + 3\tau/\rho_H) \quad (2)$$

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where p_0 is the limiting polarization at low temperature, τ is the excited lifetime of the fluorophore. The relaxation time of a rigid sphere (ρ_0) can be computed from the equation:

$$\rho_0 = \frac{3\eta V}{R \cdot T}$$

or

$$1/p = 1/p_0\,(1 + \frac{R \cdot T}{V\eta}\,\rho_0) \qquad (3)$$

where the molar volume, $V = \bar{v}_2 \cdot M_r$, and η is the viscosity of the solvent at temperature T. Fluorescence lifetimes (τ) of DnS conjugates were measured at 25°C with the same fluorimeter, but with a modified nanosecond fluorescence decay time apparatus and electronics from EG and G (Princeton NJ). A thyratron lamp, filled with N_2, at 100 lb/in^2 was used as a light source. For measuring the emission anisotropy $A(t)$, the excited light was passed through a Corning 7-37 filter and the emitted light through a Kodak Wratten 66 filter. The parallel and perpendicular components of the fluorescence decay, $F'_y(t)$ and $F'_x(t)$, were recorded on a multichannel analyzer (Model 7040, EG and G) which was interfaced to a PDP 11/34 computer and a Hewlett Packard x–y plotter. Characteristics of the excited light were obtained by replacing the sample with a Ludox suspension, the scattered light from which was recorded after removal of the emission filter and setting of the emission polarizer at 54° with respect to the direction of the excitation polarizer. The recorded fluorescence curves are distorted by the finite duration of the excited light pulse and the response time of the detection system. They were corrected and deconvoluted, using the methods of moments [13], to obtain the time fluorescence decays in order to calculate $A(t)$. The time-dependent emission anisotropy, $A(t)$, was subjected to a single or double exponential analysis to determine the rotational correlation times:

$$A(t) = A_0 e^{-t/\rho_m} \qquad (4)$$

The harmonic mean rotational relaxation time (ρ_m) was calculated from the initial slope of the semilogarithmic plot of $A(t)$ *vs* time, t (eq. (4)). The accuracy of the rotational correlation times measured was estimated to be ±0.2 ns.

3. Results

The rotational relaxation time of α can be determined from the dependence of the polarization of fluorescence on viscosity of a fluorochrome bound to the α-subunit. A Perrin plot of the determined polarization values of DnS-labelled α and α–ATP complex at pH 7.8 is shown in fig.1, yielding (ρ_H^{25}) of 18.86 ns. Different results were obtained for α at pH 6.0, yielding a relaxation time (ρ_H^{25}) of 14.5 ns, and in the presence of 0.2 mM ATP of 38.5 ns; a similar value for α-ATP at pH 8.0 of (ρ_H^{25}) of 40.0 ns was found. The lifetimes at other temperatures were computed from that at 25°C as well as the relative fluorescence intensities between 5°C and 50°C, since the lifetime generally is proportional to the intensity under these conditions. A relaxation time (ρ_H^{25}) of 14.3 ns for α at pH 6.0 and pH 8.0, calculated from the slope and intercept of fig.1 by the Perrin equation as well as from anisotropy measurements, is smaller than that expected for a rigid molecule of M_r 60 000, or even 57 000. The relaxation time of an unhydrated sphere (ρ_0) with the same M_r and partial specific volume ($\bar{v}_2$) is 28.5 ns; hence, the relaxation ratio (ρ_H/ρ_0) is 0.25. However, the relaxation ratio for α in the presence of ATP is 1.2 with ρ_H = 39.5 ns, indicating that the α-subunit in the presence of ATP behaves like a rigid, hydrodynamic, spherical molecule. Fig.2 shows the anisotropy decay curve for α–ATP at pH 7.8, and fig.3 shows that for α alone. In the

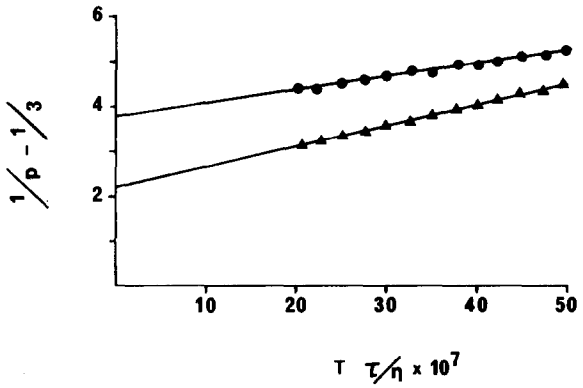

Fig.1. Perrin plot of the effect of temperature on the polarization of DnS-labelled α (○) and α–ATP (△) in 0.05 M potassium phosphate (pH 7.8), 0.15 M NaCl. The excitation (polarized) and emission wavelengths were 340 nm and 500 nm, respectively. The abscissa is in degrees nanosecond/centipoise.

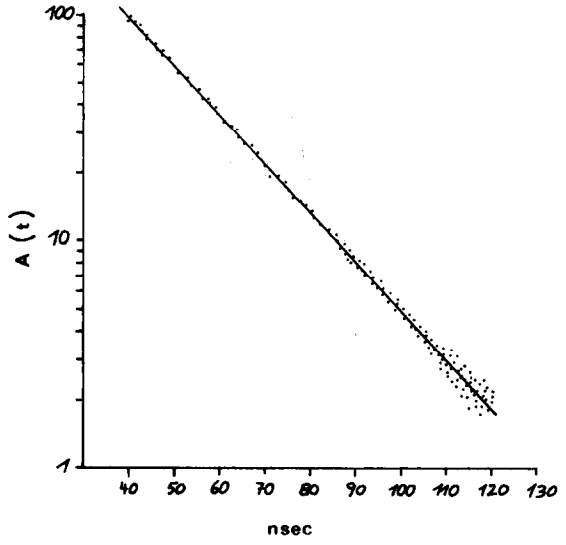

Fig.2. Decay of the fluorescence anisotropy of α-TF_1 in the presence of 2 mM ATP: τ = 28.5 ns; T = 22°C.

presence of ATP the anisotropy decays with a single exponential of $\tau_D^{25,w}$ = 28.5 ns. As a fixed approximation, the α-subunit in the presence of ATP can be considered to be spherical in shape; hence the molecular correlation volume can be determined, and it was found to be 110 000 Å^3, yielding a Stokes' radius of 32.0 Å.

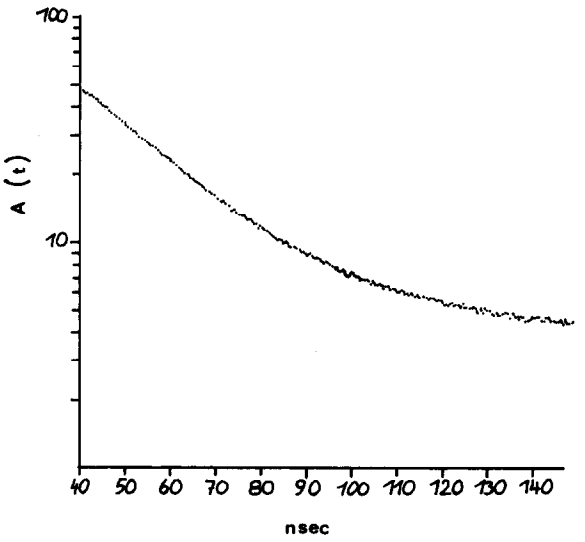

Fig.3. Decay of fluorescence anisotropy of α-TF_1 in the absence of ATP (pH 8.0). Best two-exponential fit: $A(t)$ = 1.9 $\exp^{-t/14.8}$ + 1.2 $\exp^{-t/37.2}$ ns; T = 22°C.

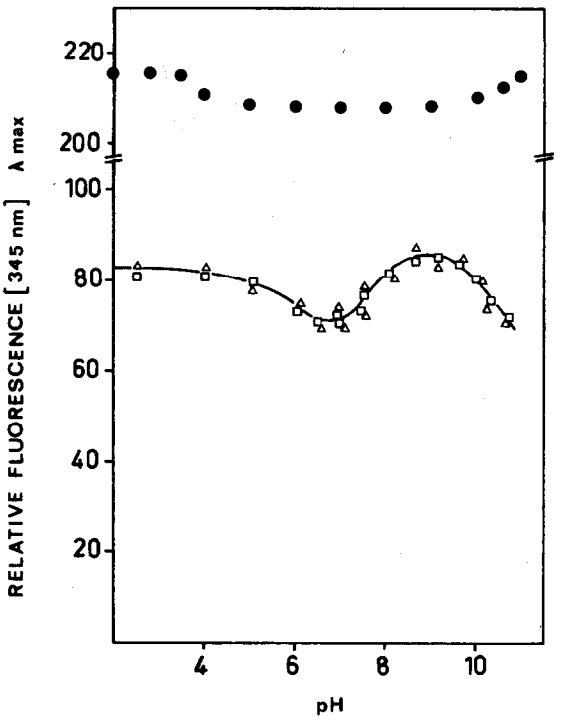

Fig.4. The pH dependence of the fluorescence and emission peaks of tryptophanyl residues in α-TF_1 at 0.18 mg/ml (○) and 0.02 mg/ml (△) in 0.05 M potassium phosphate, 22°C. Excitation was at 281 nm. The inset shows the emission peak of α-TF_1 as a function of pH.

The stability of α at various pH-values was evaluated by measuring fluorescence. At pH 10, the fluorescence intensity of unlabelled α is due to tryptophanyl emission, since the peak is shifted to the same value as observed for α in 6 M guanidinium–HCl [14] (fig.4). The fluorescence intensity, however, is quenched somewhat before the emission peak increases, possibly due to energy transfer from tryptophanyl to tyrosyl residues. The fluorescence intensity curve between pH 6.0 and pH 8.0 is biphasic. It decreases below pH 8.0, and then increases below pH 6.5. Since the first change in intensity is not accompanied by a shift in emission, it probably originates from the protonation of carboxyl groups, which are located near 1 or more of the 4 tryptophanyl residues of α [8]. The increase in fluorescence from pH 6.5 to pH 6.0 is associated with a shift in emission and, therefore, represents a structural transition. The fluorescence changes from pH 6.5–6.0 are independent of α between 0.01% and 0.0015% and presumably indicate a conformational change. This is substantiated by

anisotropy measurements. The structural transition observed by fluorescence between pH 6.0, at which reconstitution experiments normally are carried out [15,16], and pH 8.0 indicates that the acid transition involves only a limited unfolding of the α-polypeptide chain.

4. Discussion

Fluorescence depolarization and lifetime measurements can provide considerable information about size, shape and flexibility of a biological macromolecule in solution. Frictional ratios (f/f_0) for α of 1.57 have been calculated from measurements of sedimentation velocity, inelastic light scattering measurements and small angle X-ray scattering experiments [10,17–19]. This frictional ratio is rather high, resulting in an axial ratio of 6–8, if α is considered as an unhydrated, rigid ellipsoid of revolution, or if the molecule is not rigid. The time-dependent emission anisotropy, $A(t)$, is the sum of 5 exponentials for a molecule having no element of symmetry:

$$A(t) = A_0 \sum_{i=1}^{5} f_i e^{-t/\rho i} \quad (5)$$

However, in cases where the molecule can be approximated as a rigid ellipsoid of revolution, $A(t)$ (eq. (4)) decays as a sum of these exponential terms, which further reduced to 2 exponential terms if the transition dipole of the chromophore is randomly oriented. But, at times shorter than the shortest rotational relaxation time shown by a molecule, $A(t)$ can be approximated by a single exponential decay, $A(t) = A_0 e^{-t/\rho_m}$, where ρ_m is the harmonic mean rotational relaxation time. However, the harmonic mean rotational relaxation time for α-TF_1 at pH 7.8 in 0.05 M potassium phosphate, containing 0.15 M NaCl, of ρ_m = 14.3 ns is related to:

$$\frac{1}{\rho_m} = \frac{1}{A_0} \sum \frac{f_i}{\rho_i} \quad (6)$$

For a rigid, non-spherical molecule, ρ_m is larger than ρ_S, the rotational relaxation time of a rigid sphere having the same hydrodynamic volume. In contrast, local rotational motions of a fluorophore or segmental flexibility of α-TF_1 will contribute to the emission anisotropy as an exponential term with the rotational correlation time much shorter than ρ_S. However, this flexibility could arise either from unfolding, as is found with denatured proteins, or from rotational freedom between almost globular units of $M_r < 57\,000$. The relaxation ratio found for α of 0.5 is too small to account for rotational freedom. Therefore, it is necessary to invoke rotational freedom within the α-subunit of TF_1. Furthermore, it is more likely that the origin of this flexibility is due to molecular domains rather than to random unfolding, since α from TF_1 can be denatured by the same conditions which also unfold other globular proteins [20,21].

However, for α in the presence of ATP, the anisotropy decay is a single exponential, yielding hydrodynamic parameters of R = 31.0 Å and a frictional ratio $f/f_0 = R/R_0$ where:

$$R_0 = (3\bar{\nu}_2 \cdot M_r)/(4\eta \cdot N_A)$$

with ν_2 = 0.743 ml/g, obtained from the amino acid composition [4], M_r = relative molecular mass (58 900 ± 2800) (Paradies and Kagawa, unpublished), and N_A = Avogadro's number, revealing an axial ratio of 2.0, if unhydrated, and 1.5 when assuming a hydration of 0.2 g H_2O/g protein. However, the anisotropy decay is markedly non-exponential in the absence of ATP (fig.3) with the faster decay due to the flexing motion over a limiting cone angle of $\theta = 54°$, while the slower decay corresponds to rotation of the whole protein. Moreover, it should be noted, that considerable uncertainty exists in the absolute values of the axial ratio, even when calculating the shape by taking the 2 relaxation times together with the ratio of the 2 amplitudes, since the degree of hydration is not known precisely. Under this assumption, an axial ratio of 4 can be approximated.

The very fact that the anisotropy decay curve in fig.3 was non-exponential allowed us to conclude firmly that, in addition to the internal flexibility of α-TF_1, there is a definite ATP-dependent conformational change.

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