J. Biochem. **92,** 2043–2046 (1982)

Nanosecond Fluorometric Investigation of Hydrodynamic Properties of Adenosine Triphosphatase from Thermophilic Bacterium PS3[1]

Kazuhiko KINOSITA, Jr.,* Akira IKEGAMI,* Masasuke YOSHIDA,** and Yasuo KAGAWA**

Institute of Physical and Chemical Research, Hirosawa, Wako, Saitama 351, and **Department of Biochemistry, Jichi Medical School, Kawachi-gun, Tochigi 329-04

Received for publication, August 31, 1982

The soluble portion (TF_1) of proton-translocating ATPase from thermophilic bacterium PS3 was labeled with a fluorescent dye *N*-(1-pyrene)maleimide. The decay of fluorescence anisotropy of the adduct showed that TF_1 in aqueous solution was characterized by a volume of equivalent sphere of 1,120 nm^3. This value is 2.4 times the volume calculated from the molecular weight and partial specific volume, indicating a non-spherical shape and/or extensive hydration. A prolate ellipsoid with an axial ratio of 2 to 3 is suggested as a first approximation of the shape of hydrated TF_1. The presence or absence of ATP, ADP, or Mg^{2+} did not alter the volume of the equivalent sphere appreciably; the probable conformational change of TF_1 induced by these ligands does not lead to a gross alteration of its hydrodynamic properties.

The soluble portion (F_1) of proton-translocating ATPase has been identified under an electron microscope with the 90-Å particles on mitochondrial and other energy-transducing membranes (*1*). Image reconstruction from an electron micrograph of a two-dimensional crystal of thermophilic F_1 (TF_1) has shown that the molecule is hexagonal and contains a low-density region near its center (*2*). The hexagonal shape is consistent with the subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (*3*).

In order to investigate the properties of TF_1 in solution, we estimated the rotational correlation time of TF_1 using time-resolved fluorescence anisotropy decay measurement. The results suggest a non-spherical shape, in agreement with a recent study by small-angle X-ray scattering (Furuno, T., Ikegami, A., Kihara, H., Yoshida, M., & Kagawa, Y., submitted for publication).

TF_1 was obtained from thermophilic bacteria PS3 as previously described (*4*). In a typical labeling procedure, 1 μl of *N*-(1-pyrene)maleimide (PMI, obtained from Molecular Probes, Inc., U.S.A.) in acetone was added to 200 μl of TF_1 solution containing 50 mM HEPES and 0.1 mM

[1] This work was supported in part by a research grant for Solar Energy Conversion-Photosynthesis from the Japan Science and Technology Agency.

Abbreviations: TF_1, thermostable F_1 (soluble ATPase from thermophilic bacterium PS3); PMI, *N*-(1-pyrene) maleimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

EDTA, pH 7.0, at a dye/TF_1 molar ratio of 3. The mixture was incubated at 35°C for 2 h in the dark. During this period, the fluorescence intensity increased gradually from zero, indicating the adduct formation (*5*). Unreacted dye was removed by passing the sample through filter paper and then through a Sephadex G-50 column. The final dye/TF_1 ratio was determined spectrophotometrically to be 0.2–0.3. Higher dye/protein ratios during incubation and/or a longer incubation time (*e.g.* overnight at 4°C) resulted in a greater degree of labeling, but the decay of fluorescence anisotropy was unchanged.

Fluorescence decay after pulsed excitation was measured with a nanosecond fluorometer as previously described (*6*). The decay of total fluorescence intensity was multiexponential; curve fitting with three exponentials revealed that the longest fluorescence lifetimes were 160 ns at 5°C and 120 ns at 45°C. The decay of fluorescence anisotropy was also analyzed by a curve-fitting procedure (*7*), in which the three-exponential results above were assumed for the intensity decay. In the fitting, the initial 30 ns portion of the data was neglected in order to minimize the effect of blank fluorescence (unlabeled TF_1 gave weak, short-lived fluorescence, which was subtracted prior to the analysis) and the possible contribution from hydrolysis/aminolysis products of the PMI adduct (*8*).

Figure 1 shows typical decays of fluorescence anisotropy. As indicated by the dashed lines, the experimental data at each temperature could be fitted satisfactorily with a single-exponential decay. The time constant for the decay, ϕ, represents the rotational correlation time of the PMI-TF_1 adduct in solution; ϕ is related to the volume of equivalent sphere, V_e, of the adduct as follows:

$$\phi = \eta V_e / kT \qquad (1)$$

where η is the viscosity of the medium, k the Boltzmann constant, and T the absolute temperature (*9*). Values of ϕ at different temperatures gave V_e identical within experimental error, indicating that the structure of TF_1 was stable between 5 and 45°C. Table I summarizes the results obtained in various solvents. The presence of the nucleotides did not change V_e beyond the experimental error. Conformational change of TF_1 upon nucleotide binding as suggested by hydrogen-exchange kinetics (*10*) or electron microscopy (*11*)

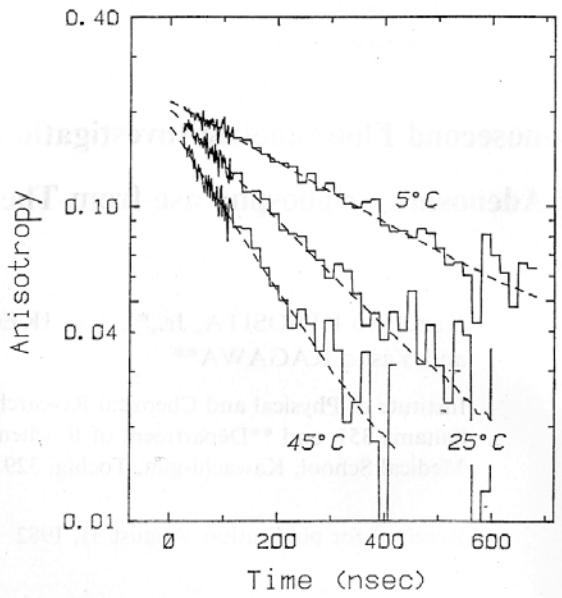

Fig. 1. Fluorescence anisotropy decay of PMI-TF_1. TF_1 was labeled with PMI as described in the text. A sample solution containing 0.37 μM PMI, 1.9 μM TF_1, 5 mM $MgSO_4$, 0.1 mM EDTA, and 50 mM Na-HEPES (pH 7.0) was excited with pulsed, polarized light at 343 nm (bandwidth 8 nm); polarized intensity components of the fluorescence were detected by two separate photomultiplier tubes through cut-off filters (transmission above 370 nm) and time-resolved by the single photon counting technique. The total intensity (not shown) and anisotropy (noisy curves) of fluorescence were calculated from the polarized components, and analyzed by a curve-fitting procedure (*7*). The dashed lines show the best-fit curves calculated on the assumption of single-exponential decay.

appears to be relatively small.

The molecular weight of TF_1 is 380,000 (*12*). If we take a value of 0.75 for the partial specific volume of TF_1 (*12*), the volume of an unhydrated TF_1 molecule, V_0, is calculated to be 475 nm^3. This is considerably smaller than V_e in Table I. In general, the difference between V_0 and V_e is attributed to hydration and/or departure of the molecular shape from a spherical symmetry (*9*). If TF_1 were spherical, V_e of 1,120 nm^3 (Table I, column 1) would imply hydration of as much as 1.0 g H_2O/g protein, which is equivalent to a hydration shell with a thickness of 1.6 nm. Though part of the water molecules may lie in the low-density region revealed in the electron micrograph (*2*), hydration to such an extent is very unlikely. In fact, an X-ray study (Furuno, T., *et al.*, unpublished) indicated that the volume of a hydrated

TABLE I. Volume of equivalent sphere (V_e) for the PMI-TF_1 adduct. All solutions contained 50 mM Na-HEPES (pH 7.0) and 0.1 mM or 1 mM EDTA in addition to the indicated substances. Average ± standard deviation (number of independent determinations).

Addition	None	5 mM NaADP	5 mM $MgSO_4$	2 mM MgATP 5 mM $MgSO_4$
Temperature range examined (°C)	5–35	5–35	5–45	5–45
V_e (nm^3)	1,120±90 (14)	1,150±110 (17)	1,180±70 (5)	1,240±90 (5)

TF_1 molecule, V_h, was 750 nm^3 in the absence of ADP and Mg^{2+}, corresponding to hydration of 0.44 g H_2O/g protein. Thus, the shape of TF_1 is not spherical. The extent of deviation from a spherical symmetry is characterized by the shape factor, V_e/V_h, of about 1.5.

The shape factor can be interpreted in terms of the axial ratio if we assume that the shape of TF_1 is approximated by an ellipsoid of revolution. Theoretical anisotropy decay for an ellipsoid of revolution consists of three exponentials (*13*), but the decay in many cases is closely approximated with an exponential function at least over one decade of decay (*9*). For a fixed molecular volume, departure of the axial ratio from one results in a slower decay (larger shape factor). The relation between the shape factor and the axial ratio, however, is not simple because the anisotropy decay depends also on the orientation of the attached dye with respect to the ellipsoidal body. Following Yguerabide (*9*), therefore, we calculated theoretical anisotropy decays for various combinations of axial ratio and dye orientation (more than 100 curves were generated by a computer), and compared the calculated decays with the experimental data. The results suggest the following: (i) Theoretical anisotropy decays for oblate ellipsoids are practically single-exponential, at least over the initial decade, irrespective of the axial ratio or of the way of dye attachment. The rate of decay depends primarily on the axial ratio and not on the orientation of the dye. If TF_1 is approximated with an oblate ellipsoid, its axial ratio will be 0.36; a hydrated TF_1 molecule will be characterized by principal semi-axes of $a=2.9$ nm and $b=c=7.9$ nm. (ii) For prolate ellipsoids, the form and the rate of anisotropy decay depend on the axial ratio as well as on the angle θ between the direction of the transition moment of the dye and the symmetry axis of the ellipsoid. A smaller θ or higher axial ratio results in a slower decay. Deviation from exponential decay is noticeable for large θ, particularly when the axial ratio is also large. (iia) For $\theta=0$, theoretical anisotropy decay is single-exponential. The shape factor of 1.5 for TF_1 then implies an axial ratio of 2.0 ($a=8.9$ nm, $b=c=4.5$ nm). This value is the lower limit for prolate TF_1, because a larger θ predicts a higher axial ratio. (iib) For $\theta=\pi/2$, the analysis yields an axial ratio of 4.3 for TF_1, which is the higher limit. Theoretical anisotropy for this case, however, deviates markedly from single-exponential, in contradiction with the experimental data (Fig. 1). Thus, the axial ratio of TF_1 must be considerably smaller than 4.3. (iic) If the orientation of the dye with respect to TF_1 is random, the axial ratio is predicted to be 3.0 ($a=11.7$ nm, $b=c=3.9$ nm). Here, the deviation from single-exponential decay is not so severe. In summary, the axial ratio of hydrated TF_1 is 0.36 if it is oblate, and between 2 to 3 if it is prolate. In either case, the maximal length on ellipsoid approximation, $2b$ or $2a$, is consistent with the maximum particle length of 18.6 nm estimated from the X-ray data (Furuno, T. *et al.*, unpublished). Since TF_1 in the two-dimensional crystal is contained in a hexagon with a diameter of 90 Å (*2*), we prefer the prolate model.

The actual shape of TF_1 cannot be a smooth ellipsoid, as evidenced by electron microscopy (*2*) and the X-ray study above. The prolate model deduced from the hydrodynamic behavior should be regarded as a gross approximation. The dimensions estimated from the fluorescence data, however, are in accord with the X-ray data as stated above. Hydrated TF_1 is considerably larger than would be expected for a compact sphere with the same molecular weight. The fact that V_e, or the overall shape, of TF_1 was rather insen-

sitive to the presence or absence of nucleotides or Mg^{2+} (Table I) is also consistent with the X-ray results, which showed a change of at most 3% in the radius of gyration upon binding of ADP or Mg^{2+}.

We thank Mrs. Keiko Nakajima for help in preparation of the manuscript.

REFERENCES

1. Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem.* **241**, 2475–2482
2. Wakabayashi, T., Kubota, M., Yoshida, M., & Kagawa, Y. (1977) *J. Mol. Biol.* **117**, 515–519
3. Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1978) *Biochem. Biophys. Res. Commun.* **84**, 117–122
4. Kagawa, Y. & Yoshida, M. (1979) *Methods Enzymol.* **55**, 781–787
5. Weltman, J.K., Szaro, R.P., Frackelton, A.R., Jr., Dowben, R.M., Bunting, J.R., & Cathou, R.E. (1973) *J. Biol. Chem.* **248**, 3173–3177
6. Kinosita, K., Jr., Kataoka, R., Kimura, Y., Gotoh, O., & Ikegami, A. (1981) *Biochemistry* **20**, 4270–4277
7. Kinosita, K., Jr., Mitaku, S., Ikegami, A., Ohbo, N., & Kunii, T.L. (1976) *Jpn. J. Appl. Phys.* **15**, 2433–2440
8. Kouyama, T. & Mihashi, K. (1980) *Eur. J. Biochem.* **105**, 279–287
9. Yguerabide, J. (1972) *Methods Enzymol.* **26**, 498–578
10. Ohta, S., Tsuboi, M., Yoshida, M., & Kagawa, Y. (1980) *Biochemistry* **19**, 2160–2165
11. Kagawa, Y., Sone, N., Hamamoto, T., Futai, M., Ohta, S., & Wakabayashi, T. (1981) in *International Cell Biology 1980–1981* (Schweiger, H.G., ed.) pp. 719–727, Springer-Verlag, Berlin
12. Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., & Ui, N. (1979) *J. Biol. Chem.* **254**, 9525–9533
13. Tao, T. (1969) *Biopolymers* **8**, 609–632