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Cold Lability and Dissociation of the F_1-ATPase from *Bacillus stearothermophilus*

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Summary: F_1 from *Bacillus stearothermophilus* dissociates at 4°C in 0.04 M TRIS-PO_4, pH 7.5-8.0, reversibly into a protein with a molecular weight of 51,000, identified as β. Concomitantly, a loss of ATP hydrolysis activity was manifested. However, heat activated F_1 in the presence of ATP and 10% (v/v) glycerol at pH 7.5 does not dissociate at 4°C and resists thermal inactivation. The weight average molecular weights and translational diffusion coefficients for the heat activated F_1 was found to be 368,000 ± 15,000 and for the cold labile enzyme after subunit dissociation to be 320,000, with diffusion coefficients of (3.58 ± 0.03) x 10^{-7} $cm^2 \cdot sec^{-1}$ and (3.28 ± 0.04) x 10^{-7} $cm^2 \cdot sec^{-1}$, respectively.

Introduction

The production of ATP in oxidative and photophosphorylation is mediated by the protonophoric action of an H^+-translocating ATPase (E.C. 3.6.1.3) (1-3). The complex is composed of two parts: one portion, designated as F_o, is an integral part of the membrane with a molecular weight of 70,000-100,000 (4-7), and the remaining peripheral part, F_1, is water soluble and can easily be detached from the membrane, has ATPase activity and a molecular weight of 365,000 ± 15,000 (8-9).

For CF_1, F_1 from mitochondria and *E. coli*, as well as from yeast, a loss of ATP hydrolysis activity has been reported (10-14), followed with a dissociation into subunits, one of which has been identified as α (14). We report here the reversible dissociation of F_1 from *Bac. stearothermophilus* at

Abbreviations used: SF_1 = the portion of the H^+-translocating ATPase which is peripheral to the membrane from *Bac. stearothermophilus*; CF_1 = F_1 from spinach chloroplasts; ECF_1 = F_1 from *E. coli*; α, β, γ, δ and ε = subunits in the order of decreasing molecular weight.

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4°C and 20°C, at low ionic strength and in the absence of ATP. Furthermore, we present evidence that heat activated F_1-ATPase from Bac. stearothermophilus in the presence of glycerol and ATP does not dissociate into subunits at 4°C and does not show any thermal inactivation, similar to TF_1 (15) but different from most mesophiles.

Materials and Methods

F_1 was prepared from Bac. stearothermophilus (strain 799, M 1359) as described in (16). Dissociation-inactivation was performed at 4°C in 10 mM TRIS-HCl, pH 7.8, containing 1 mM dithioerythreitol (DTT), 1 mM EDTA and PMSF. ATPase activity was measured by the release of P_i, which was measured according to Subbarow and Fiske (17). - The F_1-ATPase was assayed in a solution of 5 mM ATP, 5 mM $MgCl_2$, in a 40 mM Tricine-NaOH buffer at pH 8.0 in a final volume of 0.25 mL at 40°C. After 10 min the reaction was terminated by addition of 0.1 mL of 10% (w/m) trichloroacetic acid, and P_i was determined (17); and in the case where the F_1-ATPase was stored in 0.1 M K_2HPO_4, pH 7.8, by release of $^{32}P_i$ from λ-$|P^{32}|$-ATP and counting in a liquid scintillation counter (Beckmann LS 40). The purified F_1-ATPase has a specific activity of 131.5 units/mg, where one unit of activity is defined as the amount of liberated 1 µmol P_i/min/mg under the assay conditions.

Elastic and inelastic light scattering measurements. The experimental setup and its operation has been described in detail elsewhere (18, 19). The cuvette was thermostated with a Peltier element and was constant within ± 0.5°C. The experimental data were stored and processed on a PDP 11/34 computer, interfaced to the light scattering apparatus.

Separation and purification of the dissociated subunits. The dissociated F_1-ATPase at 4°C and 20°C was subjected to DEAE-sepharose-Cl,6B chromatography in the absence of ATP and glycerol in 30 mM K_2HPO_4, pH 8.0, and eluted with a linear gradient of NaCl ranging from 10 mM to 350 mM in a total of 300 mL at 20°C. Two peaks emerged, one eluting at 100 mM NaCl (main peak) and one at 250 mM salt (minor peak). The two peaks were pooled separately and rechromatographed on Ultrogel AcA22 and used for determination of the weight average molecular weight and diffusion coefficient and for SDS-polyacrylamide gel electrophoresis.

High pressure liquid chromatography (HPL) was performed on a setup purchased from Waters, Ass. (Milford, Mass., USA), using an I 250 column. The elution buffer consisted of 50 mM K_2HPO_4, pH 7.8, containing 1 mM DTT and 1 mM EDTA, applying an elution rate of 2.5 mL/min (2000 psi). The column was calibrated with proteins of known molecular weight and similar apparent partial volumes at 20°C.

SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborne (20) as a slab gel electrophoresis (Bio-Rad Laboratories), using 10% polyacrylamide and 0.1% SDS.

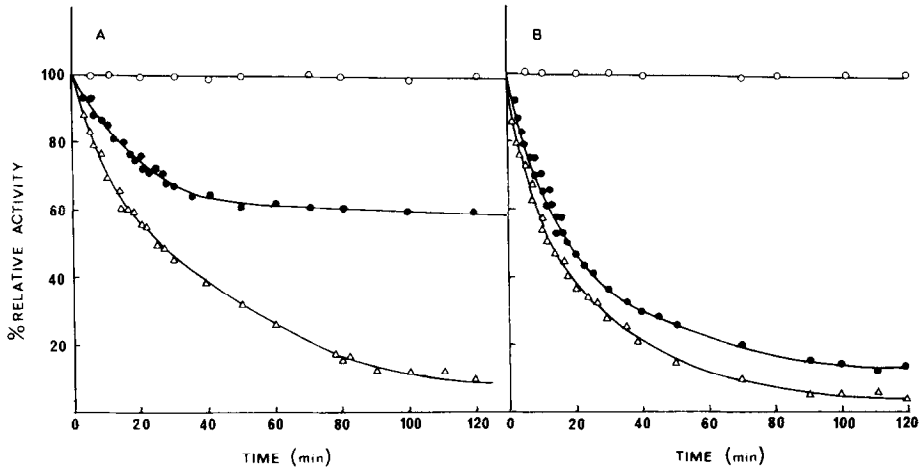

FIG. 1. Plot of the inactivation of F_1-ATPase at different initial protein concentrations at 4°C and 20°C. At zero time, the ATP concentration in the buffer of the F_1-ATPase solution was lowered from 2 mM ATP to zero ATP concentration in a final buffer of 0.05 M TRIS-Cl, pH 8.0, containing 0.1 mM EDTA and 1 mM dithioerythreitol. Enzymatic activity as a function of time was measured as described in Materials and Methods. O——O heat activated F_1-ATPase in the presence of glycerol and ATP. (A) Results obtained at a final protein concentration of 5 mg/mL (●——●) and 50 µg/mL (Δ——Δ). (B) Results obtained at a final enzyme concentration of 1 mg/mL (●——●) and 10 µg/mL (Δ——Δ).

Results

Figure 1 shows zero order plots of the inactivation of F_1-ATPase at pH 8.0 at 4°C and 20°C at various initial enzyme concentrations in the absence of ATP. It can be deduced from these data that the rate and extent of inactivation are protein concentration dependent, with the loss of ATPase activity becoming faster and more extensive at lower F_1-ATPase concentrations. Inspections of the first order plots of these data with respect to the two phases of inactivation reveals that, at lower enzyme concentrations, the fast phase accounts for a greater loss of activity. The apparent half-times of the two phases are also dependent on the F_1-ATPase concentration. However, incubation of the F_1-ATPase at pH 8.0 in the presence of ATP and 10% (w/w) glycerol prior to the inactivation studies at 4°C revealed a loss of ATPase activity of 5%, only. Furthermore, inactivation is completely abolished if the buffer at pH 8.0 contains ATP, and no temperature dependence of inactivation can be observed. But following the rate of in-

activation of F_1-ATPase in a buffer at pH 8.0, containing no ATP and with ionic strength of Γ = 0.1 M, the fast phase of inactivation follows the same trend at 4°C and 20°C, whereas at Γ = 1.0 M, the second phase of inactivation can be seen, only, independent of temperature.

The kinetics of inactivation of F_1-ATPase from Bac. stearothermophilus was correlated simultaneously with light scattering as well as diffusion measurements, revealing a biphasic nature, also, but, in addition, a decrease in weight average molecular weight from 370,000 to 320,000 which is associated with both phases of inactivation. The same change was observed for the translational diffusion coefficient. Furthermore, no change in the weight average molecular weight or in the mean diffusion coefficient, $\bar{D}$, was detected when the F_1-ATPase was in buffer solution in the presence of ATP or ATP and 10% (w/w) glycerol, or at ionic strength Γ = 1.0 M (Fig. 2). However, at elevated temperatures (20°C and 32°C), the final extent of inactivation is considerably smaller when ADP and/or ATP is present in the buffer. The half-life-time apparent for each phase is nearly identical by elastic and inelastic light and activity measurements.

The cold inactivated F_1-ATPase was subjected to HPL- and DEAE-Sepharose-Cl-6B chromatography, and the dissociated subunit as well as the remaining part of F_1 were purified. The homogeneity of the dissociated protein as well as of the remaining part of F_1-ATPase was examined by HPL-chromatography, native and SDS-polyacrylamide gel electrophoresis. The dissociated protein migrates in the native and SDS-polyacrylamide gel electrophoresis with the same K_d-value as for isolated β from F_1-ATPase. Furthermore, HPL-chromatography revealed the same retention time as β, as a weight average molecular weight of 50,000 ± 4,000 (Table 1), resulting in an effective hydrodynamic radius of R_H = 32.5 Å. For α of F_1-ATPase from Bac. stearothermophilus we observed a weight average molecular weight of 56,900 ± 4,000 and R_H = 37.0 Å. Furthermore, the isolated protein was analyzed by analytical ultracentrifugation, yielding a single symmetrical peak with $S^{o}_{20,w}$ = 2.7 ± 0.05 S, a value similar to that obtained for isolated β, whereas α under the same conditions revealed an S-value of $S^{o}_{20,w}$ =

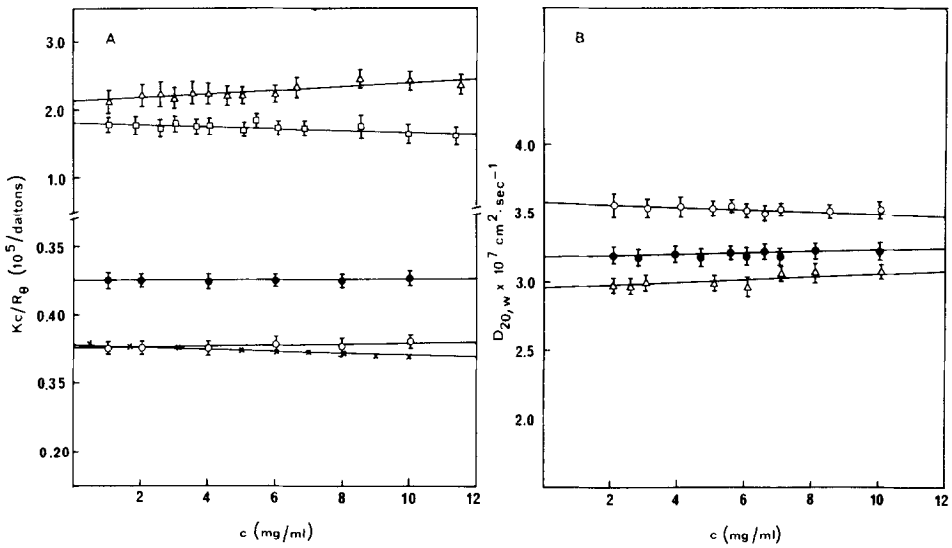

FIG. 2. (A) Zero angle extrapolation from light scattering measurements for α-SF_1 (O———O), β-SF_1 (Δ———Δ), the dissociated form of SF_1, SF_1' (O———O), native SF_1 (O———O) and SF_1 at ionic strength of Γ = 0.1 M (×———×); all at pH 8.0 and 4°C. $K = 2\pi^2 n^2 (dn/dc^2 \times (1 + \cos\theta')/\lambda^4 N$, with n the value assmed to be that of the buffer, which was determined from the differential refractive index of the buffer to pure water, and θ' the angle at which the intensity of the scattered light is measured (24.875°). R is the Rayleigh scattering factor of the protein solution minus that of the buffer, and λ is the wavelength.
(B) the corresponding diffusion coefficients, determined by inelastic light scattering.

3.47 ± 0.05 S (Table 1). The remaining part of the F_1-ATPase, after dissociation of β, yielded a molecular weight of 320,000 with a sedimentation constant of $S^{\circ}_{20,w}$ = 9.4 ± 0.3 S; all other parameters are listed in table 1.

The reversibility of the dissociation of F_1-ATPase can be demonstrated, as shown in figure 3, when the dissociation products, developed in the first phase, are re-incubated at 40°C for 1 h in the presence of 10% (w/w) glycerol or for 4 h in the absence of glycerol, but in the presence of ATP. Furthermore, reactivation of isolated, cold dissociated β-subunit with the remaining part of the F_1-ATPase to fully active F_1-ATPase is obtained by incubating the mixture at pH 8.0 in the presence of 5 mM dithioerythreitol, 2 mM ATP, and 10% (w/w) glycerol at pH 6.0. Measurements of the weight average molecular weight yielded values of 370,000 ± 10,000 at pH 8.0

Table 1. Hydrodynamic properties of the isolated fragment (P_F) after cold dissociation of F_1-ATPase from Bac. stearothermophilus

Parameter	P_f	α	β	F_1	F_1'
$M_r \times 10^4$	5.2 ± 0.3	5.69 ± 0.3	5.0 ± 0.2	37.0	32.1
$D^o_{20,w} \times 10^7$ a) $cm^2 \cdot sec^{-1}$	2.91	5.6 ± 0.2	2.90	3.54	3.21
$S^o_{20,w}$, S b)	2.70	3.45	2.70	12.6	9.4
v_2, $ml \cdot g^{-1}$ c)	0.741	0.738	0.741	0.738	0.741
f/f_o d)	1.42	1.35	1.40	1.12	1.15
R_H, Å	34.4	36.3	34.4	55.5	50.1

a) determined by inelastic light scattering (18).

b) determined by analytical ultracentrifugation (27).

c) precision densitometry (18).

d) actual frictional ratio.

in the presence of glycerol and 365,000 ± 12,000 at pH 6.0. These values are similar to those found for the active five-subunit enzyme.

Discussion

The kinetics of inactivation of the F_1-ATPase at 4°C and 20°C in the absence of ATP can directly be correlated with the dissociation of the enzyme of molecular weight 370,000 to 320,000 and 50,000, simultaneously determined by elastic and inelastic light scattering and activity measurements. The curve representing the loss of ATPase activity and dissociation into subunits in the absence of ATP at 4°C and 20°C is biphasic and very similar to a first order plot of inactivation of the F_1-ATPase at different salt concentrations in the absence of ATP. At low temperatures the final extent of inactivation is considerably higher, as is the extent of dissociation. Since the solution consists of two high molecular weight species, an active enzyme of 320,000 dalton and an active enzyme of 370,000, in the presence of the dissociated β-subunit, a preliminary assignment of the rate constant for the first

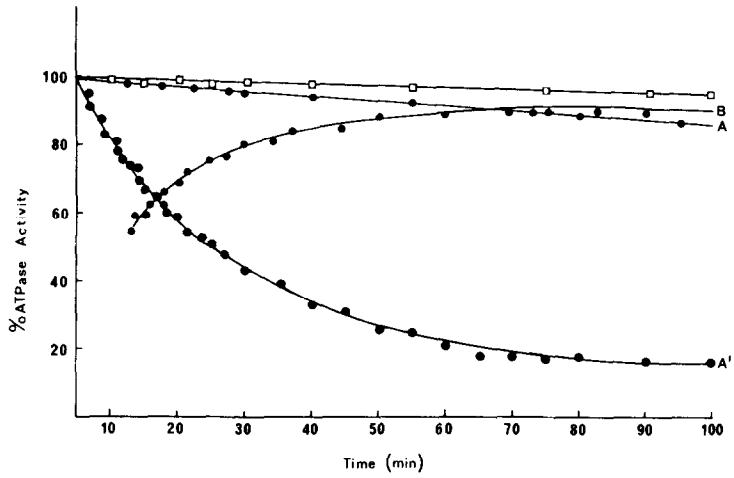

FIG. 3. Plot of F_1-ATPase activity under cold treatment in the presence (A) and in the presence (A') of ATP, and of reactivated F_1-ATPase (B) as a function of time, at pH 6.0. in the presence of 10% glycerol and 1 mM ATP. □——□ heat activated F_1-ATPase, which was subjected to cold dissociation for comparison.

biphasic curve was made, yielding a value of $k_1 = 3.05 \times 10^{-3}$ sec^{-1}, $k_{-1} = 4.3 \times 10^{-4}$ M^{-1} sec^{-1}, belonging to the dissociation from

$$F_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F_1'$$

whereas the slow phase involves the conversion of F_1 to F_1'', which is an inactive form of the F_1-ATPase that cannot be activated and has a rate constant of $k_2 = 8.0 \times 10^{-4}$ sec^{-1} and $k_{-2} = 5.0 \times 10^{-5}$ sec^{-1}. Because the mechanism of dissociation is complex, the assignment of particular steps to the phases outlined above has not been achieved for any of the coupling factors from prokaryotic sources. The half-times of the two phases are very likely functions of several of the four rate constants, at least, or six rate constants following the hysteretic concept of Frieden (21-22). However, simulation of the loss of activity under other conditions of ATP, ADP and analogs, as well as pH, at several F_1-concentrations may allow the assignment of the effect of three rate constants.

The hydrodynamic properties (Table 1), the electrophoretic mobility as well as the amino acid composition (data not shown) indicate that the dissociated subunit is β. Determinations of the molecular weights of the native, fully active F_1-ATPase from

Bac. stearothermophilus and the inactive one lead to the conclusion that one β-subunit is being dissociated, suggesting that this particular β-subunit is only weakly bound and essential for ATPase activity, also. However, the F_1-ATPase, heat activated in the presence of 10% (w/w) glycerol, does not show any thermal inactivation, and its shows no sensitivity in buffers containing no ATP with respect to ATPase activity, suggesting that two F_1 conformations are possible with different catalytic properties, as it has been shown for CF_1 in greater detail (23-24). Thus, the soluble F_1-ATPase exists in two states, a thermostable one which is cold stable and shows inhibition by iodine (25), and a cold labile one with dissociation of a β-subunit. The kinetics of transition from the thermostable state to the cold labile state is a monomolecular one which is preceded by an endothermic reaction associated with a high ΔH-value (25). Furthermore, this postulated change in the state of the F_1-ATPase could be much faster for the membrane-bound enzyme. Further work is being conducted in order to characterize the two states by means of small angle X-ray scattering as well as neutron scattering measurements. Moreover, the dissociation of F_1 from Bac. stearothermophilus is completely different from the cold lability of ECF_1 and F_1 from beef heart mitochondria which dissociate in α and (α,β), respectively, leaving particles of 320,000 and 265,000 behind with no ATPase ativity. Furthermore, the here described cold lability of F_1 in the absence of ATP is very different from the cold treatment of F_1 with ADP reported by Hachimori et al. (25), but consistent with the thermodynamic quantities of this F_1-ATPase (25, 26).

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