PHYSICAL CHARACTERIZATION OF THE RECONSTITUTED F_1-ATPase OF *ESCHERICHIA COLI* FROM THE MAIN SUBUNITS (α,β,γ)

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1. Introduction

The stoichiometry of the subunits in the F_1-ATPase is still unsettled. For the chloroplast ATPase the subunit stoichiometry appears to be of the $\alpha_2\beta_2$-type considering various physical and chemical investigations [1–4]. For the F_1-ATPase from mitochondria, both types, $\alpha_2\beta_2$ and $\alpha_3\beta_3$, have been proposed [5–7], whereas for bacterial F_1-ATPases evidence has been presented for an $\alpha_3\beta_3$ type [8–10]. However, in [11] a subunit stoichiometry of $\alpha_2\beta_2\gamma_2$ was proposed on the basis of reconstitution experiments, and convincing evidence was presented [12] for a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ for the F_1-ATPase from the thermophilic bacterium PS3.

Reconstitution of a functional coupling factor from isolated subunits of *E. coli* F_1-ATPase had spec. act. 150–170 units/mg for the 3-subunit (α,β,γ) ATPase [13] which is higher than that normally obtained with native F_1-ATPase of 90–120 units/mg [13,14].

This investigation reports the physical characteristics of the reconstituted, isolated 3-subunit complex (α,β,γ) from *E. coli* by means of elastic and inelastic light scattering.

2. Materials and methods

2.1. *Preparation of the isolated subunits*

α, β and γ were isolated from purified F_1-ATPase [14] (K12, λ) according to [13]. F_1 was dissociated in 20 mM Tricine–NaOH buffer (pH 6.0) at 2°C, containing 0.1 mM dithiothreitol (DTT), 1 mM EDTA, at 10 mg F_1-ATPase/ml, and dialyzed against a 20 mM Tricine–NaOH buffer (pH 6.0) containing 1.2 M LiCl, 0.1 mM DTT and 1 mM EDTA, for 24 h. α, β and γ subunits were isolated from the dissociated F_1-ATPase on DEAE-Sepharose C1-6B [13] at 4°C. Further purification of the 3 main subunits of F_1 was achieved by high-pressure liquid chromatography (Waters, Associate) on an I 250 and an I 60 column applying a buffer system of 50 mM K_2HPO_4 (pH 8.0), 0.1 mM EDTA and 0.1 mM DTT.

2.2. *Reconstitution of the 3-subunit ATPase*

The subunits α, β and γ (3 mg/ml each) were mixed in a total volume of 1 ml 10 mM Tricine–NaOH (pH 6.0) containing 0.2 M KCl, 0.1 mM DTT and 10% (w/w) glycerol [13] in the presence of 2 mM ATP, and subsequently stored at 37°C for 8 h. The reconstitution complex was chromatographed on Ultragel AcA44 in 50 mM Tricine–NaOH (pH 6.0) containing 0.15 M LiCl, with subsequent HPL-chromatography on an I 250 column. The specific activity of the 3-subunit ATPase was 150 units/mg, applying the assay system in [2,13].

2.3. *Physical measurements*

Elastic and inelastic light scattering measurements of the 3-subunit ATPase was performed at 20°C in the setup of [15,16] at pH 6.0 and pH 8.0, where a 50 mM K_2HPO_4 buffer was used. Refractive index measurements were made at 546 nm on a differential refractometer (Brice, Phoenix) at 20 ± 0.01°C. The value of (dn/dc) for the reconstituted enzyme was

Abbreviations: F_1, the peripheral part of the H^+-translocating ATPase; $\alpha,\beta,\gamma,\delta,\epsilon$, subunits of F_1 in order of decreasing relative molecular mass (M_r)

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found to be 0.185 ± 0.003 mg/g. The excess scattering intensity due to the protein is proportional to the apparent relative molecular mass (M_r'), the weight concentration (C_2) and of the refractive index increment, $(dn/dc)^2$, neglecting interparticle interference effects, since the size of the scatterers is much smaller than the wavelength of the light used (λ = 632.8 nm and 546.0 nm). The true relative molecular mass (M_r) is related to M_r' according to: $M_r = M_r'(1 + 2B_2M_r \cdot C_2)$, with B_2 the second virial coefficient. The light scattering instrument was calibrated (I_0) with known samples: the standard solution was 5 ml suprapure benzene and the instrument constant was determined at 4 concentrations of bovine serum albumin and ovalbumin [17].

Protein concentration was determined spectrophotometrically using $A_{280\ nm}^{0.1\%,\ 1\ cm} = 0.40$, determined by interferometric methods [15] for the 3-subunit complex, and 0.45 for α [17], 0.38 for β and 0.51 for γ.

3. Results and discussion

Relative molecular mass measurements of reconstituted 3-subunit ATPase of varying concentrations of ATP or Mg^{2+} in the reconstitution experiments were investigated, as shown in fig.1. Either the ATP or the Mg^{2+} concentration was varied, while the other was held constant at 3 mM. The weight average M_r ($\bar{M}_r$) at pH 6.0 in the absence of ATP or Mg^{2+} was found to be 46 500 ± 8500 (M_r'), and the z-$\bar{M}_r$ was 51 800 ± 6100 (M_z'). However, at 3 mM ATP or 2.5 mM Mg^{2+}, where maximal ATPase activity is obtained (fig.1A), the weight $\bar{M}_r$ was determined to 342 500 ± 17 700 (M_r') and 345 000 ± 17 000 (M_z').

The diffusion coefficient determined by inelastic light scattering [16] in the absence of ATP or Mg^{2+} was found to be $\bar{D} = (5.65 \pm 0.05) \times 10^{-7}$ cm^2/s (fig.1B). $\bar{D}$ is the mean diffusion coefficient, having the variance V, which is an index of solution polydispersity. However, $\bar{D}$ changes with concentration of ATP or Mg^{2+} at constant pH, and it revealed a final value of $\bar{D} = (3.41 \pm 0.08) \times 10^{-7}$ cm^2/s at the point of maximal ATPase activity with $\bar{V}$ = 2.5%. For non-interacting particles, small compared to K^{-1}, the average scattered intensity of species i, $\langle i_i \rangle$, is proportional to $N_iM_i^2$, where N_i and M_i are the number and molecular weight of species i, respectively. Thus $\bar{D} = \Gamma/K^2 = \Sigma(N_iM_i^2 D_i)/\Sigma(N_iM_i^2)$, which is the z-average diffusion coefficient $\bar{D}_z$. The average values of $\bar{D}$ and D_z for the reconstituted 3-subunit enzyme in the presence or absence of ATP or Mg^{2+}, of which one was held constant, are listed in table 1. Fig.2 shows plots of the translational diffusion coefficients as a function of the reconstituted, purified 3-subunit enzyme, having spec. act. 100 units/mg. Extrapolation to infinite dilution for curve A yielded $\bar{D} = (3.86 \pm 0.04) \times 10^{-7}$ cm^2/s at T = 293 K, at pH 6.0, ionic strength = 0.10 M, while

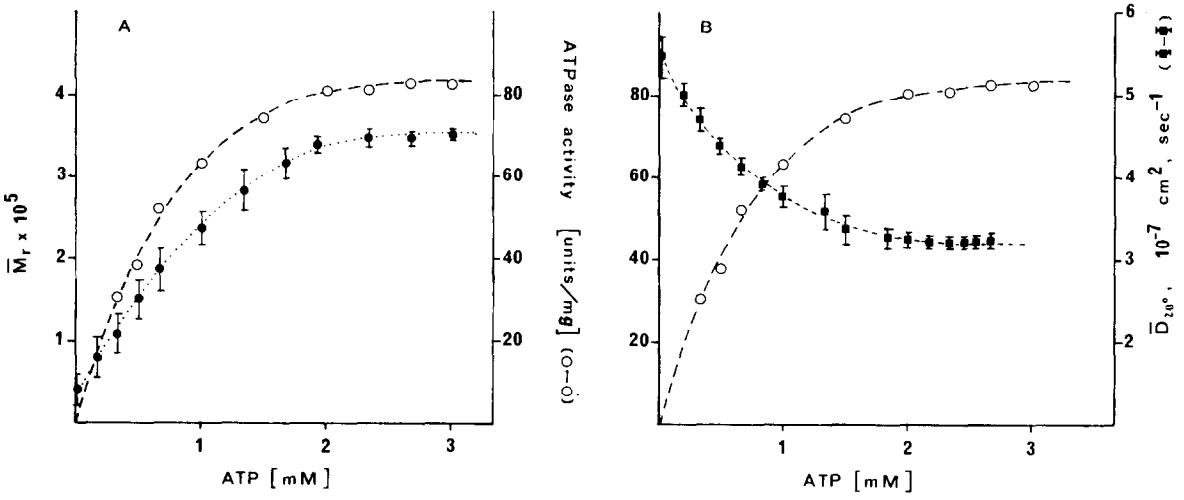

Fig.1. (A) Effect of ATP on the reconstitution of ATPase activity (○——○) and weight average M_r ($\bar{M}_r$) at constant Mg^{2+} concentration [13] and varying ATP concentration; (●——●) weight average M_r with its standard diviation. The reconstitution buffer contained 3 mM Mg^{2+}. (B) Effect of ATP on the reconstitution of ATPase (α,β,γ) activity (○——○) and mean diffusion coefficient (□——□) at constant Mg^{2+} concentration [13] and varying ATP concentration.

Table 1
Physical parameters of the reconstituted 3-subunit ATPase from *Escherichia coli*

Parameter	α,β,γ		F_1 [14,19]
	pH 6.0	pH 7.8	pH 7.8
$D^{\circ}_{20,w} \times 10^{-7}$ (cm^2/s)	3.31 ± 0.03	3.47 ± 0.03	3.61 ± 0.03
$M_r \times 10^{-5}$	3.40 ± 0.15	3.40 ± 0.11	3.68 ± 0.12
$\bar{V}_2$ (ml/g)[a]	0.734(1)	0.734(1)	0.734(0)
R_e (Å)	58.8	56.1	54.9
R_g (Å)[b]	45.3	43.2	42.3
$B_2, \bar{M}_r$ (ml . mol . g^{-2})	1.7×10^{-4}	2.5×10^{-2}	1.9×10^{-3}
f/f_o[c]	1.26–1.30	1.20–1.24	1.11
w(g H_2O/g protein)	0.69	0.57	0.58
$s^{\circ}_{20,w}$(S)	9.5	11.7	13.7
$\beta \times 10^6$[d]	2.19	2.16	2.15

[a] Determined by precision densimetry [18]
[b] Calculated according to: $R_g = k_B \cdot T/6\pi\eta_o D^{\circ}_{20,w} (5/3)^{1/2}$
[c] The degree of hydration as determined by relating the hydrodynamic volume to the volume occupied by the enzyme calculated from $\bar{V}_2$ and R_e
[d] Mandelkern-Scheraga factor [24] calculated according to $\beta = [N/16\ 700\ \pi^2]^{1/3} (f/f_o)\ \bar{V}_2^{1/3}$

the corresponding z-average translational diffusion coefficient, $\bar{D}_{z,20}$, has a value as shown by the intercept of curve B. The same results could be obtained by plotting Γ or $\bar{\Gamma}$ ($= \bar{D} K^2$) *vs* protein concentration at different scattering angles using the extrapolated Γ° (or $\bar{\Gamma}^{\circ}$) and the relation $D^{\circ} = \Gamma^{\circ} \times K^{-2}$ in order to obtain the diffusion coefficient at infinite dilution.

The reduced zero scattering angle plot from light scattering measurements is shown in fig.3. The line for each fraction is defined by the average values for the slopes and intercepts obtained from the least squares analysis of the data at the two wavelengths (λ_1 = 632.8 nm, λ_2 = 546.0 nm). The reduced infinite dilution plot for the enzyme complex also yielded straight lines representing the averages of the data of these two wavelengths. Furthermore, it should be noted that the two least squares extrapolation proce-

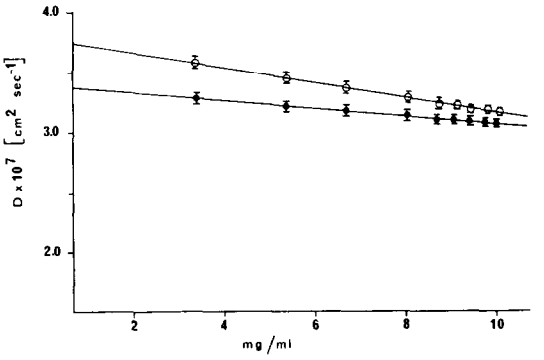

Fig.2. Plots of the translational diffusion coefficients of the reconstituted $(\alpha,\beta,\gamma)_n$ complex at pH 7.8 as a function of protein concentration, T = 293 K, ionic strength 0.1 M: (●——●) average diffusion coefficient; (○——○) z-average diffusion coefficient.

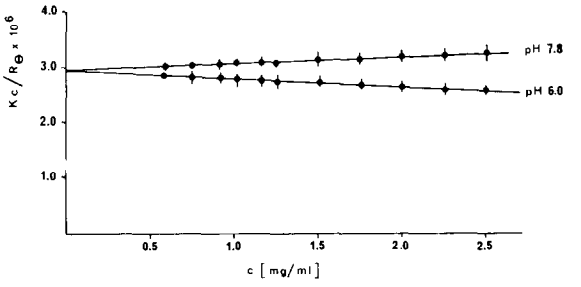

Fig.3. Zero angle extrapolation from light scattering measurements of the purified, reconstituted (α,β,γ)-ATPase at 293°C. $K = 2\pi^2 n^2 (dn/dc)^2 \times (1 + \cos\theta')/\lambda^4 N$, with n the value assumed to be that of the buffer, which was determined from the differential refractive index of the buffer, relative to pure water, and θ' the angle at which the intensity of the scattered light is measured (24.87°). R_θ is the Raleigh scattering factor of the protein solution minus that of the buffer, and λ is the wavelength.

dures, e.g., infinite dilution and $\theta \rightarrow 0^{\circ}$, yielded identical ordinate intercept values of $1/M_r$. The non-ideal behavior of the 3-subunit complex at pH 8.0 is reflected by the positive slope seen in fig.3. The weight $\bar{M}_r$ for the 3-subunit enzyme obtained is 345 000 ± 15 000, and B_2M_r was found to be 1.9×10^{-3} mol . ml . g^{-2}.

Measurements of the diffusion coefficient and M_r of the isolated 3-subunit complex after HPL-chromatography at pH 6.0, 20°C, revealed a weight $\bar{M}_r$ of 340 000 ± 15 000 and a translational diffusion coefficient of $D^{\circ}_{20,w} = (3.31 \pm 0.03) \times 10^{-7}$ cm^2/s, equivalent to an effective hydrodynamic radius of 58.8 Å (table 1). The diffusion coefficient of 3.31×10^{-7} cm^2/s is substantially lower than the one from the F_1-ATPase (table 2). However, the radius of gyration, R_g, the degree of hydration, w, and the actual frictional ratio, f/f_o (table 1), are very different from native F_1-ATPase, indicating a more extended conformation of the 3-subunit enzyme. The radius of gyration of 45.3 Å, and the one calculated from the diffusion coefficient of 43.2 Å at pH 7.8, are very close to the value determined from small angle neutron scattering [20]. The M_r reported in [20] was 312 000 ± 15 000. However, the reconstituted 3-subunit ATPase [13] as well as that from the thermophilic bacterium PS3 [9] in the presence of ATP, Mg^{2+} and 10% (w/w) glycerol give strong evidence for the subunit stoichiometry of $\alpha_3\beta_3\gamma$.

Measurements conducted at pH 7.8 (20°C) in 50 mM K_2HPO_4, containing 1 mM DTT and 2 mM ATP, revealed a different value of $D = (5.30 \pm 0.07) \times 10^{-7}$ cm^2/s for the 3-subunit enzyme with a weight $\bar{M}_r$ of 345 000 ± 17 000, whereas B_2M_r was found to be 1.9×10^{-3} mol . ml . g^{-2}. The results indicate that the contribution of the Donnan equilibrium to the second virial coefficient is larger than the contribution of the excluded volume under low ionic strength (0.05 M). But, at low pH (6.0) and low values of the average charge of the 3-subunit ATPase the excluded volume term becomes important. Therefore, at low pH the negative contribution of the charge fluctuations of the reconstitued enzyme should be small, and B_2 is closely represented by the excluded volume, whereas at pH 8.0 the Donnan term: $100\,z^2/4m_2M_r^2$ to B_2 becomes more important in addition to the excluded volume [21], with m_2 the concentration of the third component.

The results obtained for the reconstituted 3-subunit ATPase from *E. coli* according to [13] strongly suggests that the 3 main subunits isolated [13] are in a conformation in solution that allows it to reconstitute to an active particle which, with respect to ATPase activity, is almost identical to the native coupling factor. Furthermore, quasi-elastic light scattering experiments as well as M_r measurements of the reconstituted 3-subunit complex clearly indicate that the minimum subunit stoichiometry is of the $\alpha_3\beta_3$-type ($n = \bar{3}$). For comparison, reconstitution experiments of F_1 from spinach chloroplasts are shown to be of the $\alpha_2\beta_2$-type by means of small angle X-ray scattering [22]. Finally, the results obtained here cannot predict any possible arrangements of α, β and γ within the $\alpha_3\beta_3\gamma$-complex. However, the diffusion coefficient and the radius of gyration (table 1) clearly eliminate possible structure models that are not 'spherically' symmetric, such as an end-to-end aggregation of α, β and γ or side-to-side aggragation, taking the determined radii of gyration of α and β (17.25) into consideration. Furthermore, closed-ring shape aggregates can be eliminated conclusively since such a configuration should give a larger effective hydrodynamic radius and thus a lower $\bar{D}$-value than we have observed.

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