Electron microscopy of the reconstituted complexes of the F_1-ATPase with various subunit constitution revealed the location of the γ subunit in the central cavity of the molecule

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The subunit structure of the F_1-ATPase from the thermophilic bacterium PS3 was probed by comparing electron microscopic images of the subunit complexes reconstituted to contain different subunit compositions. The following structural features were observed. (1) The $\alpha_3\beta_3$ complex has a hexagonal, apparently symmetrical arrangement of masses with a central cavity. (2) The projections of the $\alpha_3\beta_3\delta$ complexes are similar to those of the $\alpha_3\beta_3$ complexes. (3) In contrast, the $\alpha_3\beta_3\gamma$ complex has an additional mass in the centre which is similar to that found in the native enzyme ($\alpha_3\beta_3\gamma\delta\varepsilon$). From these observations, it is concluded that the central mass in the F_1-ATPase is comprised mostly of the γ subunit.

F_1-ATPase; Electron micrograph; ATPase; Thermophilic bacterium

1. INTRODUCTION

Oxidative phosphorylation and photophosphorylation are catalyzed by H^+ translocating ATP synthases [1–3]. The H^+-ATP synthases isolated from various sources are very similar to each other in structure and function. The F_1-ATPase is resolved from H^+-ATP synthase as a water-soluble component which contains the catalytic part of the enzyme. Five different subunits comprise F_1-ATPases, which are designated α (M_r 55000–57000), β (M_r 50000–52000), γ (M_r 30000–36000), δ (M_r 16000–20000) and ϵ (M_r 7000–14000). The molecular weights of F_1-ATPases from various sources are 370000–400000 and have a common subunit composition of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$. Electron microscopy of the F_1-ATPases from various sources showed that six densities are arranged hexagonally around a central cavity with the seventh density partly filling this cavity [4–11]. The outer six densities are the α and β subunits with alternate arrangement [12–14]. The seventh mass tended to be obscure when the images of molecules were reconstructed by the aid of a computer from two-dimensional crystals of F_1-ATPases [8]. It seems that the central mass of individual molecules were not superimposed during computer averaging. This could happen if the location of the central mass is off-centre in the central cavity and is different in one molecule from another in the two-dimensional crystal. From the observations of trypsin-treated *Escherichia coli* F_1-ATPase, Gogol et al. suggested that the central density may correspond to the γ subunit [14]. The F_1-ATPase from a thermophilic bacterium PS3 (TF_1) is unique in that it can be reconstituted from individual subunits to form ATPase-active complexes with different subunit compositions, such as the $\alpha_3\beta_3$, $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\delta$ complexes [15–17]. This work was initiated with the premise that comparison of electron micrographs of these complexes might provide direct information on the location of the γ and δ subunits in the enzyme. During the course of this study, Yoshimura et al. reported the image analysis of the two-dimensional crystals of TF_1 and the $\alpha_3\beta_3$ complex, and concluded that the three minor subunits, γ, δ and ϵ subunits, may be arranged asymmetrically in the central portion [18]. We observed single particles rather than two-dimensional crystals of molecules to avoid the disadvantage of possible loss of information concerning the seventh mass described above. We present here direct observations that show that the γ subunit, but not other minor subunits, comprises most of the central mass by comparing the $\alpha_3\beta_3$, $\alpha_3\beta_3\gamma$, $\alpha_3\beta_3\delta$, and $\alpha_3\beta_3\gamma\delta\epsilon$ (TF_1) complex.

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Abbreviations: TF_1, F_1ATPase from the thermophilic bacterium PS3

2. MATERIALS AND METHODS

2.1. *Preparation of the $\alpha_3\beta_3$ complex*

The α and β subunits for the reconstitution of the $\alpha_3\beta_3$ complex were purified from *E. coli* harboring expression plasmids carrying

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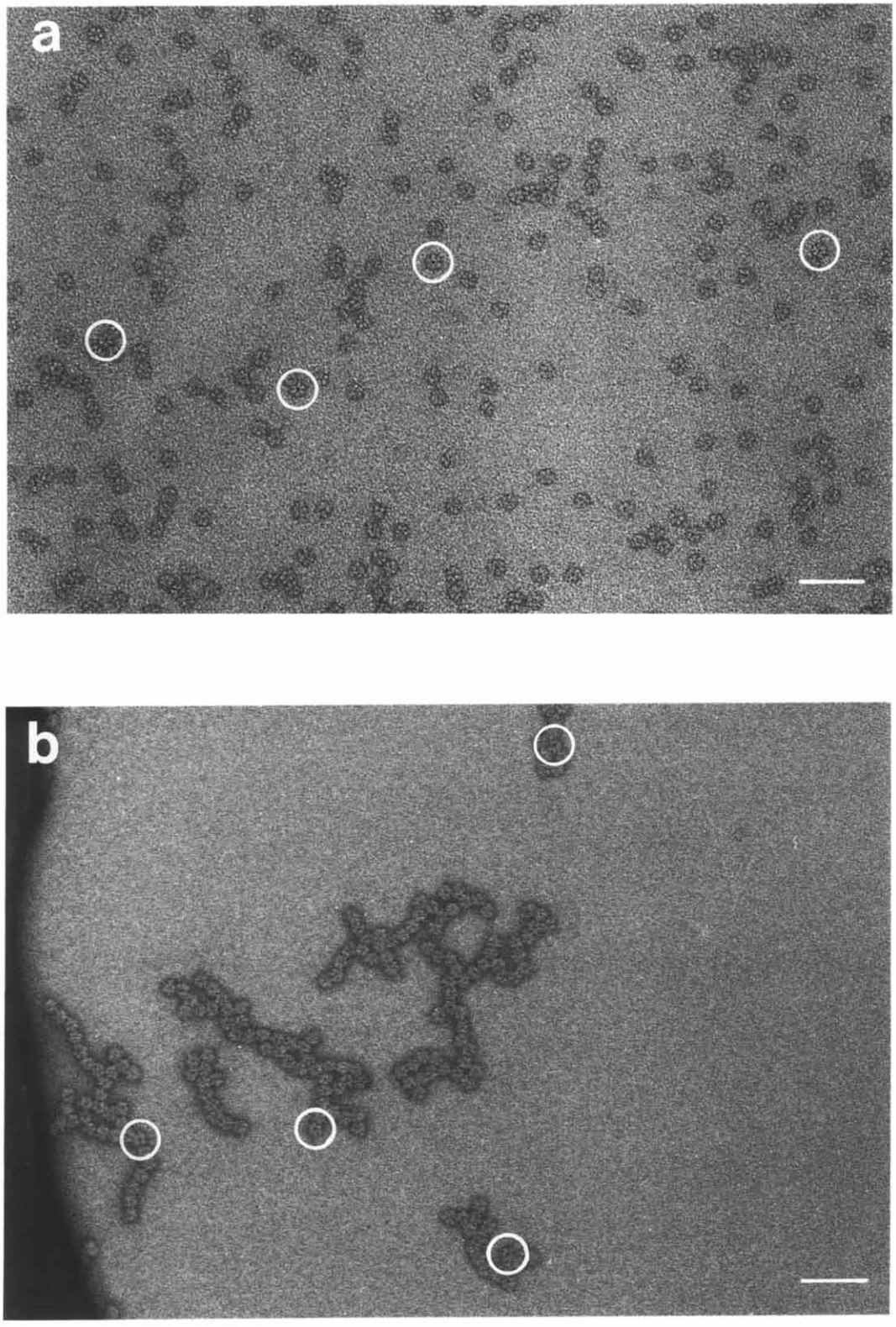

Fig. 1. Electron micrographs of the reconstituted complexes of F_1-ATPase from a thermophilic bacterium PS3; (a) the $\alpha_3\beta_3$ complexes, (b) the $\alpha_3\beta_3\delta$ complexes, (c) the $\alpha_3\beta_3\gamma$ complexes and (d) the native enzyme ($\alpha_3\beta_3\gamma\delta\epsilon$). The granularity of micrographs is due to the small amount of electron dose, use of bacitracin and underfocusing. The amount of underfocus was examined by optical diffraction and it was found no contrast reversal occurs up to 20 A resolution. The specimens were negatively stained with uranyl acetate in the presence of bacitracin and embedded in unbroken stain film suspended over holes. Micrographs were recorded with the minimal-dose technique (< 10 e/A^2). Typical views of individual particles are shown in circles. The bars in the figures represent 50 nm.

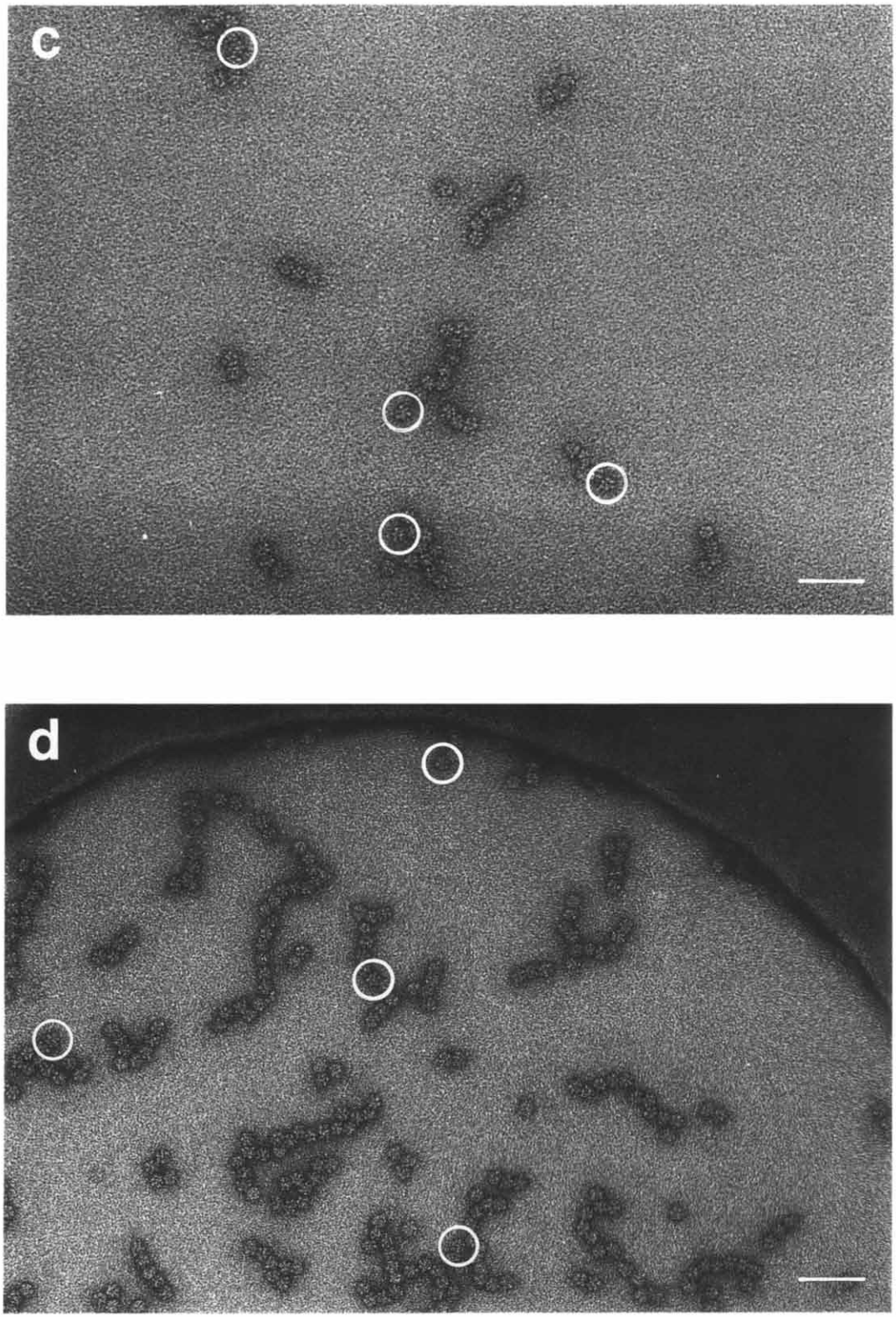

Fig. 1. continued; legend see page 112.

genes of each TF_1 subunit [19,20]. The mutant *E. coli* strain DK-8, which lacks genes of F_1-ATPase, was used to avoid possible contamination of *E. coli* F_1-ATPase subunits. Reconstitution and purification of the complexes were carried out as described in the previous paper [16].

2.2. *Preparation of the $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\delta$ complexes*

The α, β, γ and δ subunits were purified from TF_1 dissociated into subunits in 8 M urea [15]. The $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\delta$ complexes, but not the $\alpha_3\beta_3$ complex, were reconstituted from the subunits thus purified [17]. The subunits were mixed at molar ratio 3 (α):3 (β) :1 (γ or δ)

at an approximate final protein concentration of 5 mg/ml. Detailed conditions of reconstitution were described in the previous paper [21]. The complexes were purified by HPLC with a gel permeation column (G3000SW XL, Toyo Soda).

2.3. *Negative staining*

Holey carbon films were prepared on the grid according to the procedures by Fukami and Adachi [22]. The freshly purified complexes were diluted with 30 mM Tris-sulfate buffer (pH 7.5) to final protein concentrations of 10–100 μg/ml. Approximately 12–20 μl of sample solution were applied on the grid and passed through the holes of the grid by pipetting. Then a staining solution containing 2% uranyl acetate and 20–40 μg/ml bacitracin [23] was dropped on the grid once or twice. Bacitracin was used to facilitate the even distribution of staining reagent and to make the stain film over the holes more resistant to tearing induced by electron beam. After the excess solution was removed by a filter paper, the grids were dried slowly in the air. After drying, ultra-thin carbon films were formed on each surface of stain films by indirect carbon evaporation to protect the specimen from cracking and drift.

2.4. *Electron microscopy*

Specimens were examined with JEOL JEM-2000EX equipped with the minimal-dose system and the anti-contamination device. The exact magnification was determined by photographs of tropomyosin paracrystals which have 395A period [24]. Grids were scanned at a low magnification with dark illumination. After adjusting the astigmatism and focus at an adjacent area, the photograph of the target specimen area was recorded with the electron dose of about 10 e/A^2 using Mitsubishi Electron Microscope Film. This low dose condition was chosen to avoid beam damage of the specimen. Electron micrographs were surveyed with optical diffractometers to examine the amount of defocus.

3. RESULTS AND DISCUSSION

Electron micrographs of a typical field for each complex are shown in Fig. 1a–d. Complexes were sometimes linearly aggregated in a chain. Some particles showed somewhat irregular shapes, presumably corresponding to particles viewed at different angles or stained improperly. Hexagonal particles characteristic of each kind of complex are shown by the circles. The simplest complex, the $\alpha_3\beta_3$ complex, has a hexagonal, apparently symmetrical arrangement of masses which are thought to correspond to the α and β subunits (Fig. 1A). A small cavity is seen in the centre of the complex, surrounded by these masses. Although we cannot exclude other possibilities, this cavity probably extends through the entire structure and is not a simple depression of the surface of the molecule. This premise is based on the observation that the cavities are always present in the hexagonal $\alpha_3\beta_3$ complexes even if they might be a mixture of the right-side-up and the upside-down particles. Images of the $\alpha_3\beta_3\delta$ complexes are similar to those of the $\alpha_3\beta_3$ complexes in that they have central cavities (Fig. 1B). Thus the δ subunit does not seem to fill a major part of the central cavity. In contrast to the $\alpha_3\beta_3\delta$ complex, the $\alpha_3\beta_3\gamma$ complex exhibits mass (stain exclusion) in the centre of the molecule (Fig. 1C). Electron micrographs of the $\alpha_3\beta_3\gamma$ complex are almost indistinguishable from those of the intact TF_1 ($\alpha_3\beta_3\gamma\delta\epsilon$) (Fig. 1d). Thus, the four kinds of complexes examined here can be classified into two types of electron microscopic images. The $\alpha_3\beta_3\gamma$ and the $\alpha_3\beta_3\gamma\delta\epsilon$ complexes have central masses, whereas the $\alpha_3\beta_3$ and the $\alpha_3\beta_3\delta$ complexes do not. Therefore, it is concluded that the central mass in the F_1-ATPase is comprised mainly of the γ subunit. The complexes containing the γ subunit are heat stable, sensitive to azide inhibition, and show relatively strict specificity for divalent metal ions required for ATP hydrolysis. The complexes lacking the γ subunit are heat labile, insensitive to azide inhibition, and can utilize various divalent metal ions such as Cu^{2+} and Ni^{2+} [16,21]. Thus, the presence of the γ subunit in the central cavity, and its interaction with one or more α and/or β subunits, would cause a significant modification of the properties of the $\alpha_3\beta_3$ complex. The central location of the γ subunit in the molecule of the F_1-ATPase has been previously suggested from reconstitution studies of TF_1 [15], and examination of trypsin-treated *E. coli* F_1-ATPase by electron microscopy [14]. The studies using reconstituted complexes comprising a different set of subunits than reported here provide direct evidence that the γ subunit is present in the centre of a hexagonal array of the α and β subunits.

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