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Electron-microscopic studies on location of SH-groups in mitochondrial F_1-ATPase using a ferritin label

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A new approach has been suggested for electron-microscopic study of the structure of mitochondrial F_1-ATPase based on ferritin labeling. By means of sequential treatment with 2-iminothiolane and Nbs_2 we obtained a modified ferritin (NbsSPrCNH-Ft) able to react with SH-groups of proteins and to form conjugates in which the protein and ferritin are bound by disulfide bonds. An electron-microscopic investigation of the negatively stained preparations of mitochondrial F_1-ATPase, preincubated with modified ferritin, revealed such enzyme-ferritin conjugates. In case of modified ferritin, containing 360 mol SH-groups per mol protein, and F_1-ATPase, pretreated with *N*-ethylmaleimide and then with dithiothreitol, conjugates were obtained in which ferritin molecules are bound to several (as many as four) of the six protein masses, comprising a bilayer molecule of the enzyme. Taking into consideration the biochemical data on the location of accessible SH-groups (only in α, γ or ϵ subunits), it is inferred from the results obtained that one of the protein masses is a complex between β subunit and at least one of the minor subunits located partially on the molecule's external side. This indicates the nonequivalence of different copies of the major subunits. Averaged images of the particles of the F_1-F_0 complex from bovine heart mitochondria and bacteria *Micrococcus lysodeicticus* were obtained. It was found that F_0 component is bound to two adjacent protein masses of the F_1-ATPase molecule. It is suggested that this binding may be due the nonequivalency of single-type major subunits.

Introduction

F_1-ATPase (EC 3.6.1.34) is the catalytic component of the H^+-ATPase complex responsible for ATP synthesis (hydrolysis) coupled to transmembrane transport of H^+ [1,2], which is carried out by factor F_0, a membrane component of the complex. F_1-ATPases isolated from mitochondria and bacteria consist of five types of subunit in the stoichiometry 3α, 3β, γ, δ, ϵ [2–6].

An electron-microscopic investigation involving image processing of both single particles and thin crystals showed that front view projection of the mitochondrial F_1-ATPase is hexagonal approx. 10 nm in diameter [7–14]. For F_1-like ATPase from

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid.

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anaerobic bacterium *Lactobacillus casei* it was shown that six identical subunits are arranged in two layers (three in each layer) [12]. F_1-ATPases from mitochondria and *Escherichia coli* have also a bilayer structure [13–16].

So far no unambiguous data were available on the arrangement of individual subunits in the mitochondrial F_1-ATPase. X-ray diffraction data at 0.9 nm resolution were interpreted in the following way: F_1-ATPase from rat liver consists of six protein masses arranged in two layers at the vertices of a distorted triangular antiprism [13]. In the proposed model the F_1-ATPase is characterised by a pseudo twofold axis of symmetry so that different copies of the major subunits are located in different layers and thus are nonequivalent in binding to their neighbours.

On the other hand, by using electron microscopy a symmetrical model of bovine heart mitochondrial F_1-ATPase was suggested [10]. In this model three α-subunits and three β-subunits are located on different planes and are related by a threefold rotational symmetry, while a part of the central space is occupied by a seventh protein mass, which is a complex of three minor subunits (γ, δ, ϵ). In this case each of the major α- and β-subunits forms an identical set of bonds with adjacent subunits. According to the symmetrical model [10] all the α-subunits are functionally equivalent, as are all the β-subunits. The results of immunoelectron microscopy, using monoclonal antibodies specific for the α-subunits, are also consistent with arrangement of α- and β-subunits in F_1-ATPase from *E. coli* in different layers [17], but the equivalency of the subunits cannot be defined by this method.

In the present work the spatial arrangement of subunits in F_1-ATPase from bovine heart mitochondria was studied by electron microscopy employing a ferritin label for SH-groups.

Materials and Methods

The following reagents were used in the work: ferritin (Reanal, Hungary), triethanolamine, 2-iminothiolane, dithiothreitol, Nbs_2 (Serva, F.R.G.), Mops (Sigma, U.S.A.), Sephadex G-50 (Pharmacia, Sweden), *N*-[^{14}C]ethylmaleimide (24 mCi/mmol, New England Nuclear, U.S.A.).

F_1-ATPase was isolated from bovine heart mitochondria by the method of Knowles and Penefsky [18]. Submitochondrial particles from bovine heart mitochondria and membranes of the bacteria *Micrococcus lysodeicticus* were obtained as described earlier [19,20].

To obtain modified ferritin, 30 mg of ferritin (Serva) were dissolved in 2 ml of a buffer containing 100 mM triethanolamine-HCl (pH 8.5) and 0.5 mM EDTA. This solution was mixed with 0.1 ml of the freshly prepared solution of 2-iminothiolane (1 M) in the same buffer. In 40 min 0.7 ml portions of the reaction mixture were centrifuged through Sephadex G-50 column equilibrated with a buffer containing 20 mM Mops-NaOH (pH 7.5)/0.5 mM EDTA (buffer A), according to Penefsky [21]. The eluate was added dropwise under vigorous stirring to 20 ml of the 10 mM Nbs_2 solution in buffer A. The modified ferritin reaction mixture obtained was incubated for 30 min and then dialysed against water. The modified ferritin thus obtained was centrifuged at $10\,000 \times g$ for 10 min, and the pellet was dissolved in 2 ml of buffer A. The Nbs/ferritin ratio in modified ferritin was equal to 360. The content of Nbs residues in modified ferritin was determined from the absorption at 412 nm after addition of dithiothreitol to a final concentration of 2 mM, by using $\epsilon = 13\,600\ M^{-1} \cdot cm^{-1}$ [22].

The protein was assayed according to Lowry [23] with bovine serum albumin as standard.

Conjugates of F_1-ATPase with modified ferritin were obtained as follows: to 100 μl solution of F_1-ATPase (2.5 mg/ml) in a buffer containing 0.25 M sucrose/20 mM Mops-NaOH (pH 7.5)/1 mM EDTA, modified ferritin to a final concentration of 1.9–10 mg/ml was added and incubated for 0.5–4.5 h. The modification was stopped by 10-fold dilution of the reaction aliquot with the buffer containing 20 mM Mops-NaOH (pH 7.5)/1 mM EDTA.

For modification of F_1-ATPase by *N*-ethylmaleimide an aliquot of the F_1-ATPase suspension in 2 M $(NH_4)_2SO_4$ was centrifuged. The sediment was dissolved in a buffer containing 0.25 M sucrose/20 mM Mops-NaOH/1 mM EDTA (pH 7.2) (buffer B), the protein concentration being 3 mg/ml, and was desalted on a Sephadex G-50 column [21] equilibrated with buffer B. *N*-

[^{14}C]ethylmaleimide was added to the eluate to a final concentration of 2 mM, the solution was incubated for 30 min and centrifuged through the Sephadex column equilibrated with buffer B. For consecutive modification of F_1-ATPase with cold *N*-ethylmaleimide, dithiothreitol and then *N*-[^{14}C]ethylmaleimide, the enzyme (6 mg/ml) was incubated in the buffer in the presence of 2 mM cold *N*-ethylmaleimide for 30 min and then centrifuged through Sephadex, pre-equilibrated with a buffer containing 0.25 M sucrose/20 mM Tris-Mops/1 mM EDTA (pH 8). The eluate was supplemented with dithiothreitol to a final concentration of 10 mM, and after 30 min the reaction mixture was centrifuged through Sephadex, preequilibrated with buffer B. The eluate thus obtained was incubated in the presence of 2 mM *N*-[^{14}C]ethylmaleimide, and the free label was removed by centrifugation through Sephadex column. Electrophoresis in the presence of sodium dodecylsulfate was performed according to Weber and Osborn [24]. The radioactivity of the gels was counted as described in Ref. 25.

For electron microscopy the preparations were negatively stained with 5% solution of ammonium molybdate. Alternatively, some of the preparations were stained with a 1% uranyl acetate. The grids were examined in a Philips EM-400 electron microscope at a magnification of 50000 and with an accelerating voltage of 80 kV.

Results and Discussion

For the electron-microscopic studies on the location of SH-groups in F_1-ATPase we used ferritin, a protein with a molecular mass of 450 kDa, containing 17–23% hydrated ferric hydroxide and composed of 24 subunits with a molecular mass of 18.5 kDa arranged with a cubic symmetric 432. Negatively stained molecules of ferritin are easily identified on micrographs in the form of rings with an external diameter of 12 nm and an internal one of 7 nm [26].

To activate ferritin and make it specific for reaction with SH-groups, we treated the protein with 2-iminothiolane and Nbs_2. The modification of ferritin aminogroups by 2-iminothiolane enabled us to introduce additional SH-groups into the protein (the content of accessible SH-groups

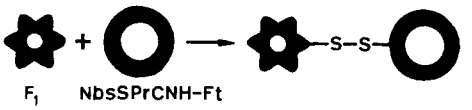

Fig. 1. Scheme of modification of F_1-ATPase accessible SH-groups by ferritin.

in native ferritin is less than 2 mol per mol protein) without any significant effect on charge of the ferritin molecule, since p*K* values of the reacting amino groups and amidine groups formed differ insignificantly [22]. The subsequent treatment with Nbs_2 allowed to obtain modified ferritin, whose reaction with the SH-groups containing proteins is accompanied by the split-off of the Nbs residues and by the formation of ferritin-protein conjugates, crosslinked by the readily cleavable disulfide bonds.

Fig. 1 shows the scheme used for the modification of accessible SH-groups of F_1-ATPase by modified ferritin. In this work we used modified ferritin preparations containing 18 and 360 mol Nbs residues per mol protein. The SH-groups of F_1-ATPase seem to have different reactivity to the various SH-reagents. Moreover, F_1-ATPase preparations isolated by various methods may differ

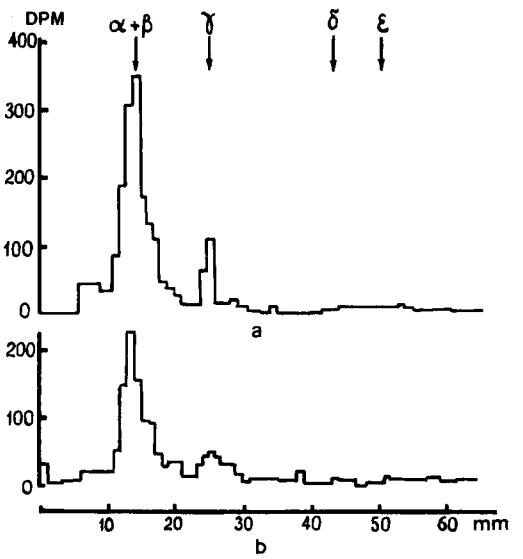

Fig. 2. Electrophoresis of F_1-ATPase modified by *N*-[^{14}C]ethylmaleimide before (a) and after (b) sequential treatment with cold *N*-ethylmaleimide and dithiothreitol. 40 μg (a) and 24 μg (b) of the protein were applied on the gel.

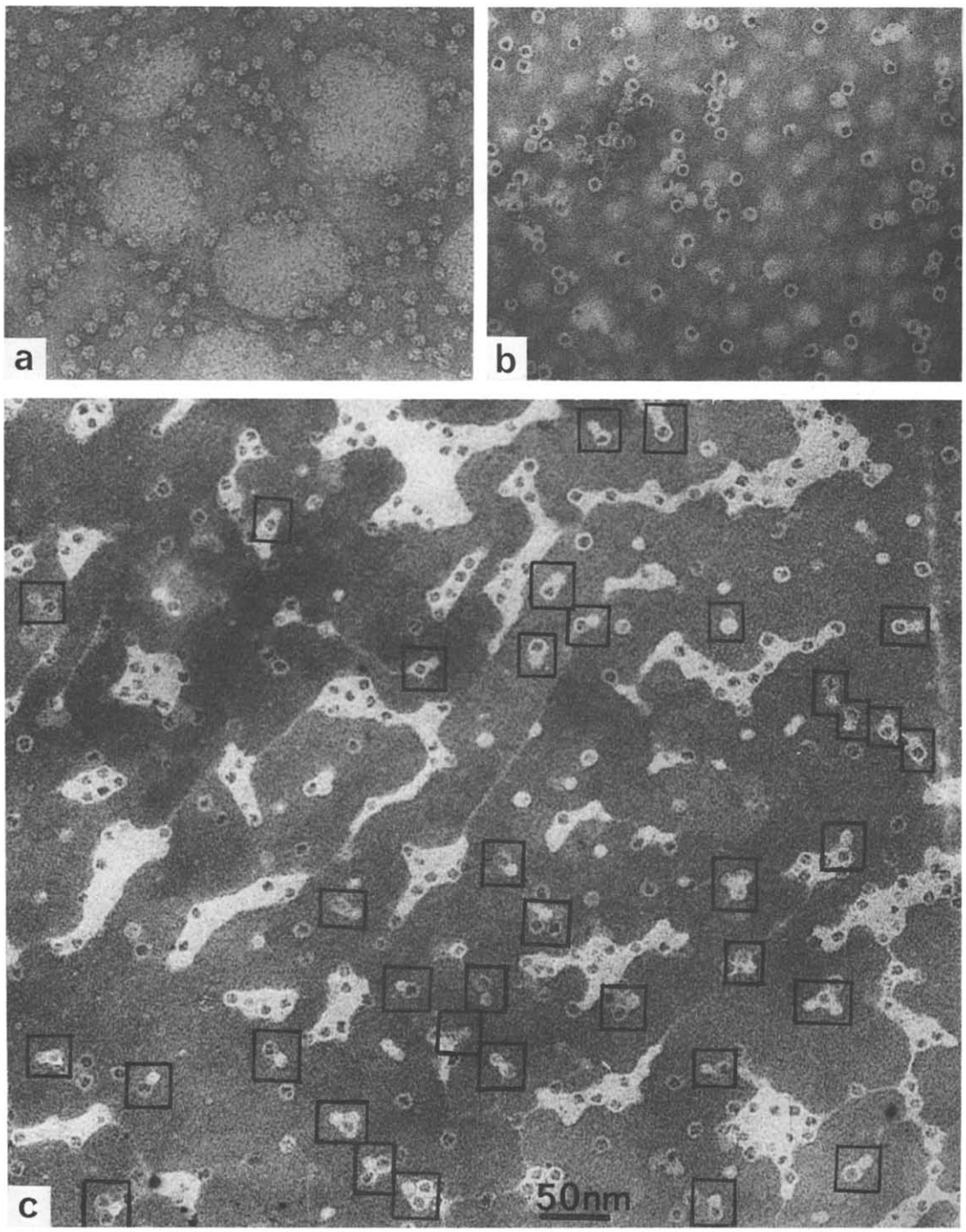

Fig. 3. General views of negatively stained preparations of F_1-ATPase (a), ferritin (b) and F_1-ATPase after preincubation with modified ferritin (c). Enzyme conjugates are framed.

in the content of accessible SH-groups. Thus Senior [27] showed that F_1-ATPase, isolated according to the method of Senior and Brooks [28], contains two SH-groups, reacting with Nbs_2 (in 15 min) and accessible to iodoacetate modification. In our preparations of F_1-ATPase isolated according to Knowles and Penefsky [18], 0.6–0.7 mol SH-groups per mol of the enzyme react with Nbs_2 or with modified ferritin in 1 min; and 1.3 and 1.6 mol in 15 and 30 min, respectively. If native F_1-ATPase is incubated in the presence of *N*-[^{14}C]ethylmaleimide, 0.5 mol of the reagent are covalently bound to the enzyme in 30 min. As seen from Fig. 2a, *N*-[^{14}C]ethylmaleimide is largely incorporated only into ($\alpha + \beta$)- and γ-subunits.

In F_1-ATPase, pretreated with unlabeled *N*-ethylmaleimide and then with Nbs_2 1.7 mol of SH-groups react within 1 min, and 2.7 and 3 mol of SH-groups per mol enzyme in 15 and 30 min, respectively. Such enzyme preparations bind 0.7 mol *N*-[^{14}C]ethylmaleimide (per mol) in 30 min. In this case too (Fig. 2b), *N*-[^{14}C]ethylmaleimide is incorporated only into ($\alpha + \beta$)- and γ-subunits, and is practically absent in minor subunits. Since the β-subunit does not contain cysteine and cystine [29,30] and by presuming that the incorporation of *N*-[^{14}C]ethylmaleimide into the enzyme's subunits reflects the distribution of accessible SH-groups among F_1-ATPase subunits, we may suggest that in both the native and the dithiothreitol-treated enzymes SH-groups accessible for modification are located on α- and γ-subunits.

Fig. 3 shows the electron micrographs of F_1-ATPase preparations (Fig. 3a), modified ferritin (Fig. 3b) and a field of F_1-ATPase molecules (Fig. 3c) after preincubation with modified ferritin, containing 18 mol Nbs residues per mol protein. F_1-ATPase conjugates with ferritin are readily revealed (some of them are framed). If F_1-ATPase is preincubated with 1 mM dithiothreitol, enzyme-ferritin conjugates disappear. F_1-ATPase does not form conjugates with ferritin even if the enzyme is mixed with native ferritin. These facts signify that conjugate formation depends on a specific reaction of modified ferritin with the accessible SH-groups of F_1-ATPase. As seen from Fig. 3c 20–30% of the F_1-ATPase molecules yield conjugates with ferritin, and there are conjugates in which one or two ferritin molecules are linked with one molecule of F_1-ATPase. The yield of F_1-ATPase-ferritin conjugates does not change if the time of the enzyme's preincubation with modified ferritin is increased from 0.5 to 4.5 h and if the modified ferritin concentration is increased.

In accordance with data in the literature [27] only two of the eight SH-groups are accessible in F_1-ATPase molecule to modification by iodoacetate and Nbs_2, while a paramagnetic analog of iodoacetate the 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidin-1-oxyl, modifies only 1 mol of the SH-groups per mol of the enzyme [31]. In this case the ESR spectrum of the modified F_1-ATPase is a sum of two types of signal: those from the strongly and those from the weakly immobilized labels. The conjugates in which a different number of ferritin molecules is bound to an enzyme molecule are presented in our preparations (Fig. 3c). This may indicate on the F_1-ATPase heterogeneity in the number of accessible SH-groups. One of the causes of such heterogeneity may be due to vicinal SH-groups in F_1-ATPase molecules. Using modified ferritin containing approx. 360 mol SH-groups per mol of the protein, as well as F_1-ATPase pretreated with unlabeled *N*-ethylmaleimide and dithiothreitol, we obtained conjugates comprising up to four accessible SH-groups. In Fig. 4 the conjugates in which F_1-ATPase is bound to ferritin by several of its protein masses, distinct in the hexagonal projection, are shown. One can see conjugates in which one (Fig. 4a) and two (Fig. 4b) ferritin molecules contact with two protein masses located at adjacent vertices. There are also conjugates in which two ferritin molecules are bound to two protein masses located at the opposite vertices (Fig. 4c) or at every other vertex (Fig. 4d) of a hexogonal 'star' and, finally, conjugates in which ferritin molecules are bound to three (Fig. 4e and f), and possibly four (Fig. 4g and h), protein masses. Ferritin molecules contact with F_1-ATPase on its external surface. As noted above, the F_1-ATPase molecule has a bilayer structure, with three protein masses in each layer. Consequently, the protein masses of both layers may be involved in conjugates formation. Our electron microscopic technique of labeling using modified ferritin have certain advantages over the antibody labeling technique. Specifically, it allowed to observe the profiles of F_1-ATPase in conjugates and

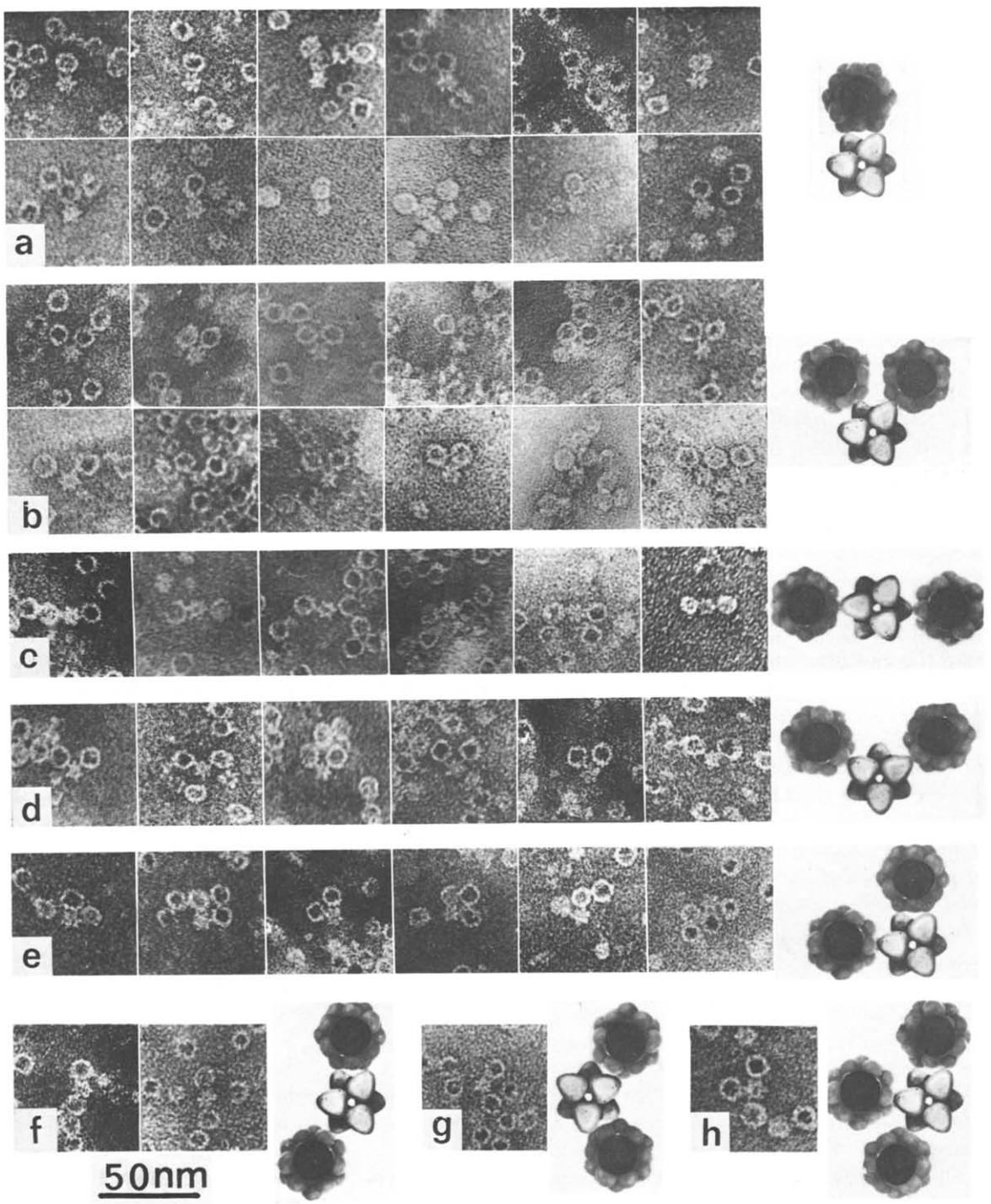

Fig. 4. Types of image of F_1-ATPase-ferritin conjugates. Models of F_1-ATPase binding with ferritin are shown on the right. The internal cavity of ferritin molecules filled with stain is shown dark.

thereby to reveal the fine features of molecule studied. Since the β-subunit does not contain cysteine and cystin residues [29,30], and SH reagents are not incorporated into the δ-subunit [32], modification of the enzyme by modified ferritin is restricted only to the α-, γ- and ε-subunits. Under our experimental conditions (Fig. 2), the SH-groups accessible to modification are most probably located on α- and γ-subunits. Accordingly, the protein masses interacting with ferritin may be the α-subunits or a complex of α- or β-subunits with a minor subunit. It follows from our data (Fig. 4) that, irrespective to the model chosen, one of the protein masses is a complex between the β-subunit and one of the minor subunits (most probably the γ-subunit). This means that part of three minor subunits (γ, δ, ε) may lie not only in the centre of the hexagonal projection of the F_1-ATPase, but also on its periphery. The fact that one of the β-subunits forms a complex with one or two minor subunits indicates the nonequivalency of individual β-subunits. Such a structural non-equivalency may be important for the function of the enzyme. Our results are in agreement with the data on asymmetrical location of the γ-subunit in the molecule of chloroplast CF_1-ATPase [33,34]. Recent electron-microscopic investigations [35] have revealed that two of the six major subunits differ from others, interacting directly with a seventh central protein mass, which occasionally may be seen in micrographs of negatively stained mitochondrial F_1-ATPase particles.

The asymmetry of the F_1-ATPase may be related to the enzyme binding with the F_0-component. To our knowledge the clear presentation of the complete complex images was never published.

In Fig. 5 we present the micrographs of the F_1-F_0 complex on the surface of submitochondrial particles (Fig. 5a) and on the surface of fragments of bacterial membranes (Fig. 5b), obtained after ultrasonic treatment of bacteria [20]. Analysing these images we have drawn attention to appearance of the F_1-F_0 complex. In each case the images of ten F_1-F_0 complex particles projecting over the membrane surface were photographically averaged, using the axis line of the 'stem' and the centre of the F_1-ATPase particle for alignment. The resulting images are shown on the right side of Fig. 5. They reveal considerable similarity. The

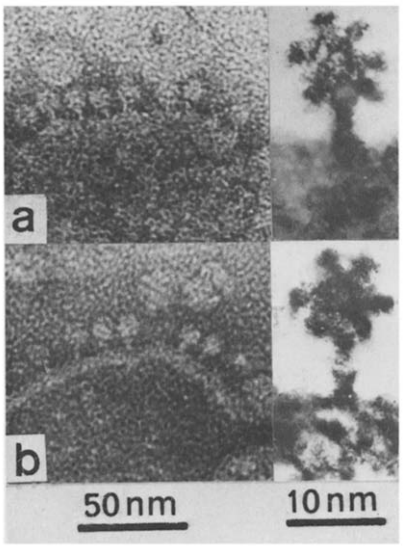

Fig. 5. Images of the complex of F_1-ATPase with F_0 on the surface of submitochondrial particles (a) and of membranes of *M. lysodeicticus* (b). The images of F_1-F_0 complex averaged by a photographic method are represented on the right.

F_1-ATPase particles are oriented by its hexagonal profile perpendicular to the membrane surface. The F_0-component is bound only to one pair of neighbouring protein masses of the F_1-ATPase, i.e., in an asymmetric manner. At present we certainly cannot conclude how closely the images observed in Fig. 5 reflect the in vivo structure of the F_1-F_0 complex; however, we believe that similarity of the F_1-F_0 particle images on the surface of both submitochondrial particles and bacterial membranes is explained by existing asymmetry of the F_1-ATPase. The observed asymmetric may be due to nonequivalence of the major subunits [36].

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