Isolation of α -subunits of factor F_1 from submitochondrial particles and the reconstitution of active ATPase from isolated α -subunits and β -subunits bound to the mitochondrial membrane

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(Received 11 March 1980/Accepted 17 July 1980)

The α -subunits of factor- F_1 ATPase are removed by extraction of submitochondrial particles with 1.75 M-LiCl, with the consequent loss of ATPase activity. ATPase activity is reconstituted by incubation of LiCl-extracted particles with purified α -subunits, and the reconstituted ATPase activity is oligomycin-sensitive. Reconstitution is enhanced by maintenance of the α -subunits in reduced form by dithiothreitol or NaBH₄ and by modification of the α -subunits by *p*-chloromercuribenzoate, iodoacetic acid or *N*-ethylmaleimide. Experiments with the mixed anhydride of ATP and mesitylenecarboxylic acid, which was previously shown to interact with the F_1 active site, localized on the β -subunits, indicate that the active site of ATPase is shielded by the α -subunits.

Factor F_1 catalyses the ATP-synthesis reaction in the oxidative-phosphorylation system in mitochondria [see reviews by Pedersen (1975) and Kozlov & Skulachev (1977)]. This enzyme has a mol.wt. of 370 000 and consists of five types of subunits, namely α (mol.wt. 55 000), β (mol.wt. 51 000), γ (mol.wt. 33 000), δ (mol.wt. 17 000) and ϵ (mol.wt. 11 000), and its subunit composition can be defined as follows: $\alpha_3\beta_3\gamma\delta\epsilon$ (Catterall & Pedersen, 1971; Knowles & Penefsky, 1972).

The δ - and γ -subunits are necessary for the attachment of factor F_1 to the membrane (Kozlov *et al.*, 1976). The active site of factor F_1 is located on the β -subunit(s) (Ferguson *et al.*, 1975; Wagenvoord *et al.*, 1977, 1979; Budker *et al.*, 1977; Esch & Allison, 1978; Drutza *et al.*, 1979; Pougeois *et al.*, 1979; Slater *et al.*, 1979), and the non-catalytic nucleotide-binding site is on the α -subunit(s) (Kozlov & Milgrom, 1980).

In some laboratories α - and β -subunits of factor F_1 have been separated and purified (Knowles & Penefsky, 1972; Senior, 1975; Verschoor *et al.*, 1977). However, under the fractionation conditions used the subunits did not remain native, and active ATPase could not be reconstructed from the purified subunits.

In the present paper we describe a method of isolating α -subunits of factor F_1 from the membrane of submitochondrial particles. The isolated α -subunits apparently retain their native structure and may be reconstituted with β -subunits bound to the mitochondrial membrane. Active ATPase, which is

oligomycin-sensitive, is formed as the result of this reconstitution.

Methods

Tris, sodium dodecyl sulphate, morpholine-propanesulphonate, NaBH₄, dithiothreitol, *p*-chloromercuribenzoic acid and carbonyl cyanide *m*-chlorophenylhydrazone were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Oligomycin, iodoacetic acid and *N*-ethylmaleimide were obtained from Serva Feinbiochemica G.m.b.H., Heidelberg, Germany. ATP and ADP were purchased from Reanal, Budapest, Hungary. [2-³H]ADP (15 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of maximum purity.

Submitochondrial particles with a specific ATPase activity of 0.4–0.7 μ mol/min per mg of protein were isolated from bovine heart mitochondria by the method of Hansen & Smith (1964). Protein was measured by the method of Lowry *et al.* (1951). The ATPase activity was measured by the pH-metric method (Chance & Nishimura, 1967), as described previously (Kozlov & Kononenko, 1975), in a medium containing 2 mM-ATP, 2 mM-MgSO₄, 3 mM-Tris/HCl buffer, pH 8.3, 0.25 M-sucrose, 2 μ M-carbonyl cyanide *m*-chlorophenylhydrazone and submitochondrial particles (0.1–2.0 mg of protein/ml). Electrophoresis of the factor- F_1 subunits was performed in a 10% polyacrylamide gel in the presence of 1% sodium dodecyl sulphate (Weber &

Osborn, 1969); 30 μ g of protein was added per tube. The gels were stained with Amido Black and scanned at 630 nm.

The α -subunits of factor F_1 were separated from the submitochondrial particles as follows. The particles (12 mg of protein/ml) were suspended in 10 mM-4-morpholinepropanesulphonate buffer, pH 7.5, containing 2 mM-ATP and 0.25 M-sucrose. LiCl up to a final concentration of 1.75 M was added to the medium, and the mixture was stirred for 30 min at 22°C. Then the suspension of the particles was centrifuged at 180 000 g for 15 min at 2°C. The pellet was washed twice, resuspended in the same buffer but without ATP (1 ml of the buffer to 10 mg of the particles), and centrifuged at 180 000 g for 20 min at 2°C. The preparation of particles obtained, which contained no α -subunits of factor F_1 (LiCl-particles), was frozen and kept for 15 days at -15°C. The specific ATPase activity of the LiCl-particles was 0.08–0.14 μ mol/min per mg of protein. This activity was inhibited by up to 95–98% by oligomycin at concentrations of 1 μ g/ml.

The supernatant obtained after the first centrifugation of the suspension of particles treated with LiCl was centrifuged for a second time at 180 000 g for 45 min at 2°C, and the pellet was removed. The supernatant obtained (50 μ g of protein from 1 mg of the initial particles) contained factor- F_1 α -subunits as the major protein component (see Fig. 1, scan 2), but also some protein contaminants. To purify the α -subunits further, the protein solution was fractionated with $(\text{NH}_4)_2\text{SO}_4$. Solid $(\text{NH}_4)_2\text{SO}_4$ (13.5 g/100 ml) was added to the protein solution at 2°C to give 20% saturation. The protein pellet was removed by centrifugation (at 20 000 g for 20 min). Solid $(\text{NH}_4)_2\text{SO}_4$ (17.9 g/100 ml) was added to the supernatant to give 50% saturation, and the solution was incubated for 60 min at 0°C. The mixture was centrifuged at 20 000 g for 20 min, the supernatant was removed, and the protein pellet was dissolved in 10 mM-4-morpholinepropanesulphonate buffer, pH 7.5, containing 0.25 M-sucrose and 4 mM-ATP (the final protein concentration was 15–20 mg/ml). The solution was rapidly heated to 50°C and incubated at this temperature for 4 min. The pellet was removed by centrifugation, and the remaining solution, containing α -subunits and only traces of protein contaminants (Fig. 1, scan 3), was frozen and kept at -15°C. The α -subunits could also be stored in freeze-dried form. The α -subunits do not possess noticeable ATPase activity.

The α -subunits obtained may be purified from traces of protein contaminants by gel filtration on Ultragel Aca 54 (column 2 cm $\times$ 80 cm). This procedure does, however, lead to a considerable loss of the total protein, apparently owing to the aggregation of the α -subunits. We used this procedure only in experiments where we had to prove

that the properties of the α -subunits observed were not determined by the presence of traces of protein contaminants.

The methods of obtaining α -subunits of factor F_1 described were used, not only with standard submitochondrial particles, but also with particles from which the protein inhibitor of ATPase had been removed (Racker & Horstman, 1967). On the whole, the results obtained with these particles did not differ from those obtained with the standard preparation of the particles.

For some unknown reason, in the cold season from October to March the α -subunits separated from the membrane by LiCl treatment contained much less protein contamination than in the rest of the year. This was observed in 2 consecutive years. All the results cited in the paper were obtained in the autumn.

Results and discussion

Philosoph *et al.* (1977) used treatment with LiCl in a procedure for the isolation of factor- F_1 β -subunits from *Rhodospirillum rubrum* chromatophores. We have now described a method involving the extraction of submitochondrial particles with LiCl that makes it possible to separate factor- F_1 α -subunits from the mitochondrial membrane.

Fig. 1 (scan 1) gives the results obtained from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of factor F_1 isolated by the method of Horstman & Racker (1970). Fig. 1 also shows the gel electrophoresis of the extract obtained as a result of treating the submitochondrial particles with LiCl (scan 2) and the gel electrophoresis of this extract after it had been subjected to further purification with $(\text{NH}_4)_2\text{SO}_4$ and to incubation at 50°C (see the Methods section) (scan 3).

From the results obtained it follows that treatment of the particles with LiCl leads to the extraction of α -subunits of factor F_1 . This conclusion is also confirmed by the following experiments. LiCl-particles were treated with chloroform as described by Beechey *et al.* (1975). Such treatment of the standard preparation of particles leads to the separation from the mitochondrial membrane of whole factor F_1 with an approximately equal content of α - and β -subunits (Beechey *et al.*, 1975). However, the factor F_1 obtained by chloroform treatment of LiCl-particles does, in the main, contain β -subunits and hardly any α -subunits (Fig. 1, scan 4).

Fig. 1 (scan 5) shows the results of gel electrophoresis of the β -subunits isolated from the factor F_1 by the method of Verschoor *et al.* (1977). The gel electrophoresis of the mixture of these β -subunits and α -subunits obtained by LiCl treatment of the particles gives a picture similar to that observed on

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of factor F_1 and isolated subunits of factor F_1 .

Scan 1: factor F_1 isolated by the method of Horstman & Racker (1970). Scan 2: factor- F_1 α -subunits obtained as a result of the extraction of submitochondrial particles with a solution of LiCl (see the Methods section). The shaded rectangles show the radioactivity of 1 mm slices of gel. To label the α -subunits, submitochondrial particles (20 mg/ml) were incubated with $100 \mu\text{M}$ of the dialdehyde derivative of [^3H]ADP (70 mCi/mmol) for 30 min in a medium containing 30 mM-morpholinopropane-sulphonate (pH 7.5) and 0.25 M-sucrose. Then 1 M-sodium borate buffer (pH 8.5) was added to give a final concentration of 100 mM, and NaBH_4 was added in two portions over 30 min to give a final concentration of 4 mM. The submitochondrial particles were washed and extracted by a solution of LiCl (see the Methods section). After electrophoresis of the extract, the gel was cut into slices 1 mm in thickness, and the slices of gel were placed in scintillation vials containing 3 ml of 1.5% solution of sodium dodecyl sulphate and incubated for 48 h at 55–60°C. Then 17 ml of dioxan scintillator (Kozlov & Milgrom, 1980) was added to

electrophoresis of factor F_1 (cf. scans 1 and 6, Fig. 1).

Previous results obtained by us (Kozlov & Milgrom, 1980) indicated that the dialdehyde derivative of ADP, formed on treatment of ADP with NaIO_4 , modifies the nucleotide-binding site of isolated or membrane-bound factor F_1 , which is located on the α -subunit(s), and does not affect the active site, which is located on the β -subunit(s). Experiments with particles preincubated with the dialdehyde derivative of [^3H]ADP showed that the protein separated from the membrane as a result of LiCl treatment of the particles contains a radioactive label (Fig. 1, histogram in scan 2).

From the results obtained, it may be concluded that the treatment of the particles with LiCl under the conditions mentioned above causes the separation of factor- F_1 α -subunits from the particles.

The incubation of LiCl-particles with added α -subunits results in the restoration of ATPase activity (Fig. 2, curve 1). The ATPase activity measured both at the intermediate stages (every hours) and also at the end of the reconstruction was completely sensitive to oligomycin. ATPase activity was inhibited by oligomycin ($1 \mu\text{g}/\text{ml}$) up to 95%.

Even more impressive results in reconstructing ATPase activity can be achieved if the α -subunits are pretreated with dithiothreitol (Fig. 2, curve 2) or NaBH_4 (Fig. 2, curve 3) before adding them to the LiCl-particles.

It is known (Senior, 1975) that disulphide bonds may be formed in the α -subunits of factor F_1 . The results obtained (Fig. 2, curves 1, 2 and 3) testify to the fact that for the successful reconstruction of the α -subunits with LiCl-particles the formation of disulphide bonds in α -subunits should be prevented. This conclusion is also confirmed by the results of experiments in which the α -subunits reduced by NaBH_4 were treated with thiol-blocking reagents that prevent the formation of disulphide bonds during the reconstruction. Thus, according to the data in Fig. 2, the α -subunits reduced by NaBH_4 and treated with *p*-chloromercuribenzoate (curve 4), iodoacetic acid (curve 5) or *N*-ethylmaleimide (curve

the vials, and the radioactivity was measured on a Nuclear Chicago mark 2 counter. Scan 3: factor- F_1 α -subunits purified by fractionation with $(\text{NH}_4)_2\text{SO}_4$ and incubation at 50°C (see the Methods section). Scan 4: factor- F_1 β -subunits isolated from LiCl-particles by the method of Beechey *et al.* (1975). Scan 5: β -subunits isolated from factor F_1 by the method of Verschoor *et al.* (1977). Scan 6: a mixture of α -subunits, obtained by extracting particles by LiCl, and β -subunits isolated by the method of Verschoor *et al.* (1977).

Fig. 2. Reconstitution of ATPase activity by incubation of LiCl-particles with factor- F_1 α -subunits

Curve 1: reconstitution of LiCl-particles with the control preparation of α -subunits. The α -subunits were twice precipitated in the cold by solid $(\text{NH}_4)_2\text{SO}_4$ (60% saturation) and dissolved in 10 mM-4-morpholinepropanesulphonate buffer, pH 7.5, containing 0.25 M-sucrose. This solution of α -subunits was added to the suspension of LiCl-particles (20 mg of protein/ml), in the ratio 50 μg of α -subunits/mg of particles. The incubation of LiCl-particles with the factor- F_1 α -subunits was performed at 22°C. At the intervals indicated on the abscissa, small samples were taken from the incubation medium and added to the medium for measurement of ATPase activity. Curve 2: before the addition of the α -subunits to the LiCl-particles, the α -subunits were treated with 1 mM-dithiothreitol for 15 min at 22°C. Curve 3: before addition to the LiCl-particles, the α -subunits were treated with 1 mM- NaBH_4 in 0.1 M-sodium borate buffer, pH 8.0, containing 0.25 M-sucrose at 22°C. After 15 min a further portion of NaBH_4 medium was added, and the mixture was incubated for another 15 min. The reduced α -subunits were salted out by solid $(\text{NH}_4)_2\text{SO}_4$. The protein pellet formed was separated by centrifugation, dissolved in 10 mM-4-morpholinepropanesulphonate buffer, pH 7.5, containing 0.25 M-sucrose, and added to the LiCl-particles. Curve 4: before addition to the LiCl-particles, the α -subunits were successively treated with NaBH_4 medium and 0.1 mM-*p*-chloromercuribenzoate (for 1 h at 22°C). Curve 5: before addition to the LiCl-particles, the α -subunits were successively treated with NaBH_4 medium and

6) reconstruct ATPase activity better with LiCl-particles than do the α -subunits that had not been treated with the thiol-blocking reagents (curve 3).

Fig. 3 shows that the preincubation of LiCl-particles with the mixed anhydride of ATP and mesitylenecarboxylic acid affects the reconstruction of ATPase activity with LiCl-particles and α -subunits. According to the results obtained by us previously (Drutza *et al.*, 1979; Kozlov *et al.*, 1979), this derivative of ATP completely inhibits the ATPase activity of isolated factor F_1 , modifying the active site, which is located on the β -subunit(s), and does not have any effect on the ATPase activity of the submitochondrial particles. We explained the resistance of the particles' ATPase to the mesitylenecarboxylic acid derivative of ATP by the fact that the active site of membrane-bound factor F_1 is immersed in the mitochondrial membrane and is protected by the membrane proteins from hydrophilic inhibitors (Kozlov & Chernyak, 1976; Kozlov & Skulachev, 1977; Kozlov *et al.*, 1979).

Fig. 3 (curve 1 and inset) shows that the mesitylenecarboxylic acid derivative of ATP inhibits the ATPase activity of LiCl-particles. This result indicates that the active site of ATPase, which is located on the β -subunit(s) of factor F_1 (Ferguson *et al.*, 1975; Wagenvoort *et al.*, 1977, 1979; Budker *et al.*, 1977; Esch & Allison, 1978; Drutza *et al.*, 1979; Pougeois *et al.*, 1979; Slater *et al.*, 1979) is shielded in the particles by the factor- F_1 α -subunits. The separation of the α -subunits from the particles as a result of LiCl treatment makes the active site accessible to the ATP derivative. The absence of complete inhibition of LiCl-particles by the ATP derivative (Fig. 3, inset) appears to be because part of the ATPase activity of the LiCl-particles is determined by a small fraction of the factor F_1 in which the α -subunits have not been depleted or not been completely depleted.

The above suggestions are confirmed by the experiments in which the reconstruction with factor- F_1 α -subunits of LiCl-particles preincubated with the mesitylenecarboxylic acid derivative of ATP was studied. As shown in Fig. 3, the addition of α -subunits to LiCl-particles preincubated with the ATP derivative results in only a negligible increase in ATPase activity (curve 1), whereas the reconstruction of ATPase activity with α -subunits and the control preparation of LiCl-particles is accompanied by a 5-fold increase in ATPase activity (curve 2).

5 mM-iodoacetic acid (for 1 h at 22°C). Curve 6: before addition to the LiCl-particles, the α -subunits were successively treated with NaBH_4 medium and 5 mM-*N*-ethylmaleimide (for 1 h at 22°C).

Fig. 3. Inhibition of the reconstruction of ATPase activity with LiCl-particles and α -subunits of factor F_1 as a result of the treatment of LiCl-particles with the mixed anhydride of ATP and mesitylenecarboxylic acid. Inset: inhibition of the ATPase activity of LiCl-particles by the mesitylenecarboxylic acid derivative of ATP (0.5 mM). Curve 1: LiCl-particles were preincubated with the mesitylenecarboxylic acid derivative of ATP (0.5 mM) for 40 min, and then α -subunits were added (shown by the arrow). Curve 2: LiCl-particles were incubated for 40 min without the mesitylenecarboxylic acid derivative of ATP, and then α -subunits were added. For the conditions of reconstruction see the legend to Fig. 2. α -Subunits treated with 1 mM-dithiothreitol were used (see the legend to Fig. 2).

The reconstruction rate and the final ATPase activity in the reconstructed system do not change as a result of the presence of 2 mM- $MgSO_4$ or 0.5 mM-EDTA in the incubation medium. On the other hand, the process of reconstruction is completely blocked by adenine nucleotides, e.g. ATP, ADP and $[\beta\gamma\text{-imido}]\text{ATP}$ (Fig. 4, cf. curves 1 and 2). It is noteworthy that inhibition of the reconstruction by

Fig. 4. Effect of nucleotides and their analogues on the reconstruction of ATPase activity with LiCl-particles and α -subunits of factor F_1 .

Curve 1 (●); without additions. Curve 2: 1 mM-ATP (□), 2 mM-ADP (▽) or 0.25 mM- $[\beta\gamma\text{-imido}]\text{ATP}$ (■) was present in the incubation medium from the very beginning of reconstruction. Curve 3: from the beginning of reconstruction for 70 min without additions, and after 70 min 0.5 mM-ATP (○) or 2 mM-ADP (▲) was added. Curve 4 (Δ): from the beginning of reconstruction for 70 min without additions, and after 70 min the mesitylenecarboxylic acid derivative of ATP (0.5 mM) was added. In all experiments α -subunits pretreated with $NaBH_4$ were used (see the legend to Fig. 2).

the adenine nucleotides is observed even when ADP or ATP is added to the incubation medium of LiCl-particles with α -subunits, not right at the very beginning, but after some time (Fig. 4, curve 3).

The mechanism by which the adenine nucleotides block the reconstruction process still remains unclear. It can only be noted that the effect of ADP and ATP differs greatly in its mechanism from the effect of the mesitylenecarboxylic acid derivative of

ATP. As distinct from ATP or ADP, when the mesitylenecarboxylic acid derivative of ATP is added to the incubation medium 70 min after the beginning of reconstruction, it does not prevent it from continuing: the reconstruction rate remains the same, and the final ATPase activity of the reconstructed system falls only by 25% (Fig. 4, curve 4). Comparing the results obtained on adding the mesitylenecarboxylic acid derivative of ATP to the LiCl-particles before the beginning of reconstruction (Fig. 3, curve 1) with those obtained in the experiment when the ATP derivative was added to the system after the beginning of reconstruction (Fig. 4, curve 4), it may be concluded that the initial stages of reconstruction already result in the shielding of the active site of factor F_1 from the ATP derivative.

All in all, the results obtained in the present paper testify to the fact that the α -subunits play an important part in the mechanism of functioning of factor F_1 associated with the mitochondrial membrane. The established fact that the active site of ATPase is shielded by the α -subunits is in good agreement with the scheme of mitochondrial ATPase functioning suggested earlier (Kozlov, 1975; Kozlov & Skulachev, 1977). According to the mechanism suggested, the non-catalytic nucleotide-binding site of factor F_1 , which is located on the α -subunit(s) (Kozlov & Milgrom, 1980), carries out a relay transfer of the substrates from the matrix to the catalytic site, which is located on the β -subunit(s) (Ferguson *et al.*, 1975; Wagenvoort *et al.*, 1977, 1979; Budker *et al.*, 1977; Esch & Allison, 1978; Drutza *et al.*, 1979; Pougeois *et al.*, 1979; Slater *et al.*, 1979) in the depths of the mitochondrial membrane (Kozlov & Chernyak, 1976).

We thank Professor Z. A. Shabarova and Dr. N. I. Sokolova for kindly providing the preparation of the mixed anhydride of ATP and mesitylenecarboxylic acid, Professor V. P. Skulachev for discussion of the results and valuable criticism, and Mrs. Glenys Ann Kozlov for translation and preparation of the paper.

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