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ASYMMETRICAL DISTRIBUTION OF THIOL GROUPS INVOLVED IN ATP-$^{32}P_i$ EXCHANGE ON MITOCHONDRIAL MEMBRANES

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Summary - The use of mitoplasts, that is mitochondria devoid of outer membrane oriented as normal mitochondria, and of sonicated vesicles, the membrane of which is inside-out has shown that the thiol groups involved in the process of ATP synthesis are on the matrix face of the mitochondrial membrane : carboxypyridine disulfide (CPDS) a thiol reagent that cannot penetrate across hydrophobic membranes does not inhibit the ATP-$^{32}P_i$ exchange catalyzed by mitoplasts, while 5,5'-dithio-*bis*-(2-nitrobenzoate), which penetrates more readily, can completely inhibit this exchange. In contrast, both reagents react similarly with inside-out vesicles. The nature of the component of the ATPase-ATP synthase complex to which this thiol group may belong is discussed.

Previous studies have shown that the number of titrable thiol groups increases during the synthesis of ATP catalyzed by intact pig heart mitochondria in oxidative phosphorylation (1). Specific thiol reagents inhibit the mechanism of oxidative phosphorylation at different levels, P_i or ADP entry and ATP synthesis (cf. review 2). Although thiol groups play an essential role in the mechanism of oxidative phosphorylation their exact location remains an open question. Griffiths and coworkers recently suggested that reduced lipoic acid was involved in the process (3). Thiol groups have been titrated in F_1-ATPase (4) and an ATP-dependent dithiol was detected in pig heart F_1-ATPase (5).

In the present work, we have tried to determine if the thiol groups involved in oxidative phosphorylation could belong to one of the protein components of the ATPase-ATP synthase complex. The structure of this complex is asymmetrically oriented in the mitochondrial membrane, the factor F_1 being located on the matrix side (6). By comparing the effect of a non penetrating thiol reagent, carboxypyridine disulfide (CPDS) (7,8) to that of 5,5'-dithio-*bis*-(2-nitrobenzoate) (DTNB) which is more permeable on the ATP-$^{32}P_i$ exchange catalyzed by submitochondrial particles of opposite polarities, we have been able to localize the thiol groups involved in the mechanism of ATP synthesis in a non polar area on the matrix side of the mitochondrial membrane. The peptide chain to which it may belong will be discussed, taking into account

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the fact that these thiol groups become more accessible to DTNB in particles which have been depleted of F_1 with urea and then reconstituted.

MATERIAL AND METHODS

Mitochondria were prepared from pig heart by a procedure derived from that of Crane *et al.* (9) as previously described (10). Purified mitoplasts were obtained as published by Maïsterrena *et al.* (11). ETP (inverted electron transfer particles) and F_1-ATPase were purified and assayed as before (12). To obtain particles depleted of F_1-ATPase the ETP were treated with urea, using a slightly modified technique described by Racker and Horstman (13) : the ETP are diluted with 0.25 M ice-cold sucrose at a protein concentration of 10 mg per ml. An equal volume of a solution containing 4 M urea, 4 mM EDTA, 100 mM Tris-sulfate, pH 8.0, freshly made up, is added. The mixture is maintained at 0°. After exactly 30 min the suspension is centrifuged at 50,000 rpm in a L2 Beckman ultracentrifuge (rotor 65). After 15 min, the pellets are first rinsed with and then resuspended in 0.25 M sucrose with 10 mM Tris-HCl, pH 7.4. The suspension is recentrifuged as above. After this washing, the urea-treated particles (ETP-U) are kept in 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4 at a concentration of 20 mg protein per ml, as small aliquots in liquid nitrogen. Fatty acid-free bovine serum albumin was prepared according to Chen (14), starting with bovine serum albumin, fraction V, obtained from Sigma.

Rebinding of F_1-ATPase to urea-treated particles was carried out by incubating 200 µg F_1-ATPase with 1 mg particles and 2 mg defatted bovine serum albumin in 0.22 ml of 0.1 M sucrose, 0.45 mM ATP, 0.1 M Tris-sulfate, 20 mM $MgCl_2$, pH 7.4 for 10 min at 20° and then 30 min at 0°. Aliquots of 100 µl of this mixture were used to determine the ATP-$^{32}P_i$ exchange activity. When indicated, F_1-ATPase was pretreated with carboxypyridine disulfide (CPDS) or 5,5'-dithio-*bis*-(2-nitrobenzoate) (DTNB) using the following method. F_1 (1 mg protein) which has been kept at 2° in 2.3 M ammonium sulfate, 1 mM EDTA, 2 mM ATP, 10 mM Tris-sulfate pH 7.5 is spun down in an Eppendorf centrifuge ; the pellet is dissolved in 0.2 ml 40 mM Tris-sulfate, 1 mM EDTA, 2 mM ATP, 1 mM DTNB or CPDS, pH 7.5 and kept at 30° for 15 min. F_1-ATPase is separated from the incubation medium by precipitation in 2.5 M ammonium sulfate. After centrifugation, the pellet is washed with 2.5 M ammonium sulfate, 2 mM ATP, 40 mM Tris-SO_4, 1 mM EDTA pH 7.4, centrifuged again and finally dissolved in 0.25 M sucrose, 2 mM ATP, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4.

ATP-$^{32}P_i$ exchange activity was determined as described by Conover *et al.* (15) in 1 ml of a medium containing : 10 mM $MgSO_4$, 10 mM ATP, 20 mM phosphate (K), 10^6 to 2×10^6 cpm $^{32}P_i$ and submitochondrial particles (0.3 to 1.5 mg protein) with the pH adjusted to 7.4 with Tris base. After 10 min at 30°, the exchange was stopped by adding 100 µl of 35 % perchloric acid. After centrifugation the $|^{32}P|$-ATP formed was estimated in the supernatant fraction after it has been extracted with isobutanol-benzene in the presence of ammonium molybdate, in acidic conditions to remove the remaining inorganic phosphate (16).

RESULTS AND DISCUSSION

A - *A comparison of the inhibition of ATP-$^{32}P_i$ exchange by CPDS, a non penetrating thiol reagent and by DTNB, a penetrating one, in submitochondrial particles of opposite polarities.*

It has been proved previously that the particles used here have opposite

polarities. The orientation of the mitoplast membrane, *i.e.* mitochondria devoid of outer membrane, is identical to that of the inner membrane *in situ* while the ETP are mainly inverted (17,18). When the ATP-$^{32}P_i$ exchange activity is measured in the presence of increasing amounts of DTNB or CPDS, a striking difference appears between mitoplasts and ETP. Both reagents react similarly with ETP (Fig. 1B). A maximal inhibition of about 50 % is obtained when the concentration of either reagent reaches 0.5 mM. On the contrary, CPDS remains without effect on the ATP-$^{32}P_i$ exchange activity of mitoplasts while DTNB can almost completely inhibit this exchange (Fig. 1A). It should be noted that, in both cases, the ATP-$^{32}P_i$ exchange is completely abolished in the presence of 1 µg oligomycin per mg protein (not shown on the figure). The experiment has been reproduced with three different preparations of mitoplasts and of ETP.

This experiment demonstrates that at least two populations of thiols are involved in ATP synthesis. They cannot be reached from the outer face of inner membrane (mitoplasts) by the non permeant CPDS. In contrast, since DTNB can completely inhibit the ATP-$^{32}P_i$ exchange from the outer face of inner membrane, it means that a population of thiols must be located in a non polar area accessible to DTNB. The fact that both CPDS and DTNB, inhibit in an identical manner but only partially the ATP-$^{32}P_i$ exchange from the inner face of inner membrane (ETP) suggests that the thiols affected must be in a polar area accessible to both reagents or even pointing towards the matrix. The partial inhibition (50 %) seems to indicate that this other population would not be directly involved in ATP synthesis but could rather affect it by changing the conformation of the enzyme, modifying the catalytic site.

Abou-Khalil *et al.* have shown that CPDS can prevent ATP synthesis in intact pig heart mitochondria by blocking specifically the access of phosphate to the ATP synthesis compartment (7). The apparent contradiction between their results and ours can be explained by the fact that we used mitoplasts instead of mitochondria. Mitoplasts are prepared by swelling mitochondria in the presence of phosphate and are more permeant ; on the other hand, the rate of ATP-$^{32}P_i$ exchange in mitoplasts or sonicated vesicles is about ten times lower in the conditions used here where the energy comes from the hydrolysis of added ATP than in the case of mitochondria oxidizing for example, succinate. Therefore, here, the rate of phosphate entry even if inhibited by the reagents is not the limiting factor ; this is why the CPDS that cannot cross the mitoplast membrane does not inhibit the ATP-$^{32}P_i$ exchange.

B - Effect of pretreating F_1-ATPase with DTNB or CPDS before reconstitution with F_1-depleted particles, on the ATP-^{32}Pi exchange activity

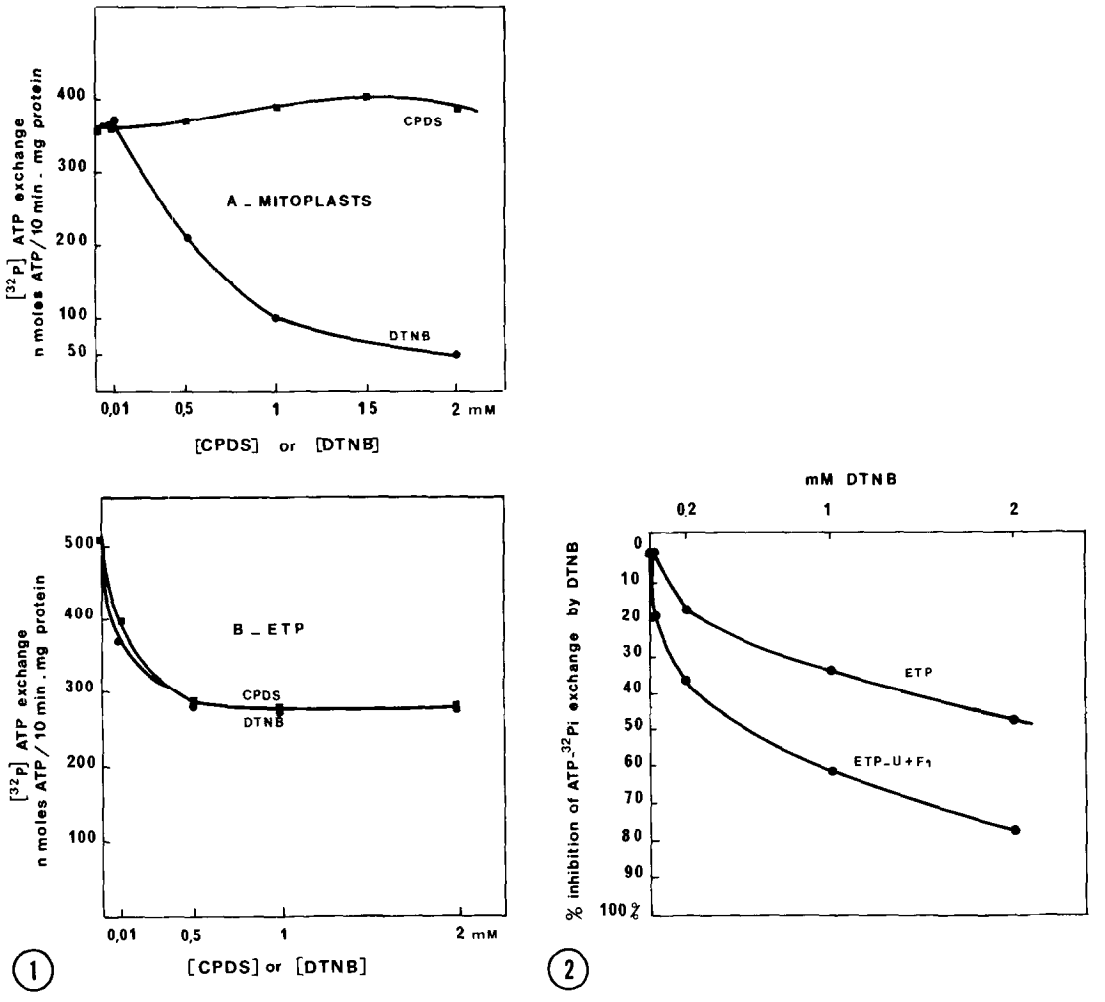

Fig. 1 - Comparative effects of DTNB and CPDS on the oligomycin-sensitive ATP-$^{32}P_i$ exchange activity of mitoplasts and sonicated vesicles (ETP).

Mitoplasts (3.2 mg protein) or ETP (3.4 mg protein) were incubated in 0.2 ml 0.25 M sucrose, 10 mM Tris-HCl pH 7.5 in the presence of the indicated concentrations of DTNB or CPDS for about 30 min at 0°. ATP-$^{32}P_i$ exchange was measured after addition of 100 µl of these particles to 0.9 ml of the ATP-$^{32}P_i$ exchange medium as described in Material and Methods.

Fig. 2 - Inhibition by DTNB of the ATP-$^{32}P_i$ exchange catalyzed by ETP freshly prepared or reconstituted (ETP-U + F_1).

Sonicated vesicles (ETP) or F_1-depleted particles reconstituted with F_1-ATPase (ETP-U + F_1) are prepared as described in Material and Methods. ETP or ETP-U + F_1 (0.8 mg protein) are preincubated with DTNB in the presence of 2 mg bovine serum albumin and the ATP-$^{32}P_i$ exchange activity is determined as in Fig. 1. In the absence of DTNB, the exchange activities are respectively 1185 and 262 nmoles $|^{32}P|$-ATP per 10 min per mg protein for ETP and ETP-U + F_1.

The identical inhibition of ATP-$^{32}P_i$ exchange induced by DTNB or CPDS in the case of the ETP which are inside-out, and which therefore expose the F_1-ATPase to the external medium (6) could be related to a direct modification of F_1-ATPase by these thiol reagents. Indeed, it has been shown previously that isolated F_1-ATPase reacts in a similar manner with DTNB or CPDS and that these compounds allow in the presence of ATP the formation of an internal disulfide bridge inside the F_1 molecule (5). To test whether the formation of this bridge could inhibit the ATP-$^{32}P_i$ exchange activity, F_1-ATPase has been treated with CPDS or DTNB before reconstitution with F_1-depleted particles. The ATP-$^{32}P_i$ exchange obtained with these particles, after reconstitution, has been compared to that obtained with particles reconstituted with F_1-ATPase not previously modified by thiol reagents. The results have shown that the pretreatment of F_1 with CPDS or DTNB does not markedly inhibit the ATP-$^{32}P_i$ exchange activity after reconstitution of F_1-ATPase with F_1-depleted particles. Besides, this pretreatment of F_1 neither affects the ATPase activity, nor changes the full sensitivity of the reconstituted particles towards oligomycin (1.4 µg/mg protein), whether it is measured on the ATP-$^{32}P_i$ exchange or on the rate of ATP hydrolysis. However from this experiment, it cannot be concluded that the thiol groups of F_1 do not take any part in the ATP synthesis process, since it cannot be proved that, during the reconstitution of ATP synthesis with F_1-depleted particles, the disulfide bridge formed during the reaction of F_1 with CPDS or DTNB (5) is not reduced again.

C - Sensitivity to DTNB of F_1-depleted particles reconstituted with F_1-ATPase

The effect of DTNB on the ATP-$^{32}P_i$ exchange in reconstituted particles ETP-U + F_1 is very much increased (Fig. 2) as compared to that of ETP ; it is very similar to the DTNB-induced inhibition obtained with mitoplasts.

This experiment indicates that part at least of the thiol groups involved in the ATP synthesis must be located at the junction between F_1 and the membrane factor F_0. It seems unlikely that it could be reduced lipoic acid as suggested by Griffiths (3) unless this lipoic acid is tightly bound to a protein component ; otherwise, the lipoic acid, being lipid soluble, could be reached equally well from both sides of the mitochondrial membrane. Moreover, Senior (19) has shown that the modification of OSCP by thiol reagents does not affect the ATP-$^{32}P_i$ exchange, and Brooks and Senior (20) have found an absence of cystein residues in the protein inhibitor of F_1. All these facts are in favour of the location of the -SH groups involved in the ATP synthesis mechanism being at the level of the membrane factor F_0 but these thiol groups would be oriented towards the matrix face of the mitochondrial inner membrane.

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