Inhibitory Chemical Modifications of F_1-ATPase: Effects on the Kinetics of Adenosine 5′-Triphosphate Synthesis and Hydrolysis in Reconstituted Systems†

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ABSTRACT: The purified, soluble F_1-ATPase was modified by several covalently reacting inhibitors, either known or considered to bind to the active site bearing β-subunit, to cause partial inhibition up to 99%. The modified enzyme was then reconstituted in the presence of OSCP (oligomycin sensitivity conferring protein) with submitochondrial particles (SMP) almost completely (>99%) denuded of active F_1-ATPase and was assayed for oligomycin-sensitive ATPase and oxidative phosphorylation activities. The inhibitors used were 1-fluoro-2,4-dinitrobenzene (FDNB), *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCD), quinacrine mustard (QM), 5-(dimethylamino)-naphthalene-1-sulfonyl chloride (dansyl-Cl), 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), and *N*,*N*′-dicyclohexylcarbodiimide (DCCD). The SMP reconstituted with unmodified F_1 exhibited oxidative phosphorylation and oligomycin-sensitive ATPase (in the presence of uncouplers) activities as high as 500 nmol min^{-1} mg^{-1} and 8 μmol min^{-1} mg^{-1}, respectively. The systems reconstituted with F_1 modified to cause various degrees of inhibition with FDNB, EEDQ, CMCD, QM, and dansyl-Cl exhibited the same degree of inhibition of oxidative phosphorylation and oligomycin-sensitive ATPase activities as the inhibition of the ATPase activity of the modified F_1 before reconstitution. The systems reconstituted with FSBA-modified F_1 showed the following relative degrees of inhibition: oxidative phosphorylation > oligomycin-sensitive ATPase of particles > ATPase of soluble F_1. In contrast, the systems reconstituted with DCCD-modified F_1 showed much greater inhibition of oligomycin-sensitive ATPase than of oxidative phosphorylation activity. In the case of DCCD, experiments with [^{14}C]DCCD showed (a) that, up to at least 2.5 mol of [^{14}C]DCCD/mol of F_1, the label was incorporated only in the β-subunit and (b) that reconstituted systems containing per mole of modified F_1 ≥1 or ≥2 mol of [^{14}C]DCCD exhibited oxidative phosphorylation activities respectively 50 and 20% of the control SMP reconstituted with unmodified F_1. Regardless of the nature of the inhibitor used (e.g., EEDQ, FSBA, or DCCD), all the systems reconstituted with F_1 modified to cause various degrees of inhibition exhibited the same apparent K_m^{ATP} in oligomycin-sensitive ATPase assays and the same apparent K_m^{ADP} and $K_m^{P_i}$ in oxidative phosphorylation assays. The apparent K_m^{ADP} values of the reconstituted systems (including the control SMP reconstituted with unmodified F_1) were 4–5 times that of "intact" SMP, and the apparent $K_m^{P_i}$ values of reconstituted systems containing modified F_1 were twice that of systems containing unmodified F_1. The significance of the above results regarding the mechanisms of ATP synthesis and hydrolysis by SMP is discussed.

Recent studies have indicated that in isolated F_1-ATPase the individual catalytic sites (three per molecule of F_1) are functionally active for ATP hydrolysis and that uni-site ATP hydrolysis at an F_1 to ATP molar ratio of 3 is a very slow process ($v = 10^{-4}$ s^{-1}) (Cross et al., 1982; Grubmeyer et al., 1982). At high ATP concentrations, when all three catalytic sites are presumably operating, this rate is increased 10^6-fold to $V_{max} = 600$ s^{-1} (Cross et al., 1982). It has been shown that this dramatic site–site catalytic cooperativity is primarily due to a 10^6-fold increase in the rate of product release from one site upon substrate binding to a second site (Cross et al., 1982). A similar mechanism has been postulated for ATP synthesis in which energy-promoted substrate (i.e., ADP plus P_i) binding to one site is considered to facilitate product (i.e., ATP) release from another site (Gresser et al., 1982). Thus, Boyer and co-workers have proposed an alternating site model for ATP synthesis in which the three catalytic sites on F_1 participate in (a) energy-promoted binding of ADP plus P_i at one site, (b) isoenergetic interconversion of tightly bound ADP plus P_i to tightly bound ATP at the second site, and (c) energy-promoted release of tightly bound ATP at the third site [Gresser et al., 1982; see also Hatefi et al. (1982)].

Thus, it was of mechanistic interest to see in reconstituted systems how ATP synthesis and hydrolysis are affected when isolated F_1-ATPase is inhibited to various extents at one or more sites by reagents capable of covalent and stable modification of the enzyme. For this purpose, F_1-ATPase was purified, treated with various covalently reacting inhibitors, freed from excess reagent by gel filtration, assayed to determine the degree of ATPase activity inhibition, then combined with submitochondrial particles deficient in F_1, and assayed again for oxidative phosphorylation and oligomycin-sensitive ATPase activity. Similar studies were done previously, with Nbf-Cl[1] as the covalent inhibitor, and the results showed a greater degree of inhibition of ATP hydrolysis than synthesis (Steinmeier & Wang, 1979; Kohlbrenner & Boyer, 1982). However, a problem with this reagent is that the Nbf moiety,

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[1] Abbreviations: SMP, submitochondrial particles; ASU particles, SMP treated with ammonia, Sephadex, and urea to remove F_1-ATPase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CMCD, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; dansyl-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; DCCD, dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FDNB, 1-fluoro-2,4-dinitrobenzene; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; OSCP, oligomycin sensitivity conferring protein; QM, quinacrine mustard; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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which appears first to react with a tyrosyl residue at the active site of F_1 (Ferguson et al., 1975), can migrate to other positions or even be removed. This possibility introduced some uncertainty in the results, which seemed to suggest different mechanistic paths for ATP synthesis and hydrolysis. The possibility of Nbf migration or removal agrees with our experiments with this reagent, which showed that Nbf-inhibited F_1 recovered considerable ATPase activity after reconstitution [similar results have been obtained by Dr. J. H. Wang (private communication)].

The covalent inhibitors we have studied were CMCD, EEDQ, FDNB, dansyl-Cl, QM, FSBA, and DCCD. Among these, F_1 modifications by the first five reagents resulted in reconstituted systems exhibiting comparable degrees of inhibition of ATP synthesis and hydrolysis (oligomycin sensitive). However, particles reconstituted with FSBA- or DCCD-modified F_1 discriminated between ATP synthesis and hydrolysis. The former showed much greater inhibition of ATP synthesis than hydrolysis, while the latter exhibited opposite characteristics, i.e., much greater inhibition of ATP hydrolysis than synthesis. In the case of DCCD, it was ascertained with the use of [^{14}C]DCCD that the inhibitions involved the stable binding of $\geq$1 mol of DCCD/mol of F_1. After reconstitution, the F_1 so modified was capable of ATP synthesis at about 50% the control rate.

Materials and Methods

Preparation of F_1-Depleted SMP. Heavy beef heart mitochondria (HBHM) were isolated (Hatefi & Lester, 1958), homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris–acetate, pH 7.5, adjusted to a protein concentration of 60 mg/mL, frozen in liquid nitrogen in 20-mL batches, and stored at −70 °C. One batch of HBHM was thawed, diluted at 0 °C with 4 volumes of cold 0.75 mM EDTA, and used for the preparation of ammonia-treated "A particles" according to Higashiyama et al. (1975). The final pellet was suspended at 55–60 mg of protein/mL in the column buffer (75 mM sucrose, 250 mM KCl, 2 mM EDTA, 30 mM Tris–sulfate, pH 8.0) used for the preparation of ASU particles. The suspension of A particles (300–350 mg) was loaded on a Sephadex G-50 (coarse) column, and the procedure of Racker & Horstman (1967) for the preparation of ASU particles was followed precisely. The final pellet was suspended in 0.25 M sucrose at 25–35 mg of protein/mL and stored frozen at −70 °C in small aliquots. The yield of ASU particles was 10–15 and 40–50%, respectively, from HBHM and A particles.

F_1-ATPase was prepared according to Senior & Brooks (1970) with deletion of the heating step, and OSCP was prepared according to Senior (1971). Reconstitution of F_1-deficient SMP (ASU particles) with F_1 and OSCP was carried out essentially according to Steinmeier & Wang (1979). The buffer contained 0.25 M sucrose, 50 mM Tris–acetate, pH 7.5, 2 mM ATP, and 2 mM EDTA, and the final protein concentrations per milliliter were 5 mg of ASU particle, 1.2–1.7 mg of F_1, and 0.03–0.1 mg of OSCP. The minimum amount of OSCP required for maximum reconstituted activity depended on the preparation of ASU particles. The reconstituted particles were either used immediately or washed once with the above buffer by centrifugation and resuspension in order to remove free F_1. Washing caused a small decrease in the assayed activities. Protein was estimated by the method of Lowry et al. (1951). ATPase (Stiggall et al., 1979) and oxidative phosphorylation (Hatefi et al., 1982) were assayed as before. The ATPase assays were conducted at 30 °C in the presence of either 1.7 μg of F_1 or 10 μg of particle protein/mL. The latter assay system also contained 10 μM rotenone and 3 μM CCCP. CCCP was added to the ATPase assays in order to be able to compare the oligomycin-sensitive ATPase activities of the reconstituted systems, since various degrees of coupling in SMP affect their oligomycin-sensitive ATPase activities. However, our results showed that the reconstituted systems exhibited very little stimulation of ATPase activity upon addition of an uncoupler to the assay medium. Oligomycin sensitivity of the reconstituted ATPase activities was routinely checked by addition of 25 μg of oligomycin/mg of particle protein. Oxidative phosphorylation assays were conducted at 30 °C in the presence of 0.2 mg of particle protein/mL and, unless otherwise stated, 0.4 μg of oligomycin/mg of particle protein. The latter reactions were initiated by the addition of succinate. The specific activities at 30 °C are reported as micromoles per minute per milligram of F_1 or particle protein for ATP hydrolysis and nanomoles per minute per milligram of particle protein for ATP synthesis by oxidative phosphorylation. The F_1 preparations used had ATPase activities at pH 7.5 in the range of 54–60 μmol min^{-1} (mg of protein).$^{-1}$ Membrane potential changes were monitored at 30 °C by the absorbance change of oxonol VI at 630 minus 603 nm essentially as described elsewhere (Yagi et al., 1983). The reaction mixtures contained 0.25 M sucrose, 50 mM Tris–acetate, pH 7.5, 1.9 μM oxonol VI, 0.2 mg of particle protein/mL, and, where indicated, 0.4 μg of oligomycin/mg of particle protein. Membrane potential formation was induced by addition of 10 mM succinate.

Hexokinase, lactic dehydrogenase, pyruvate kinase, phosphoenolpyruvate, EEDQ, QM, CMCD, FDNB, and dansyl-Cl were obtained from Sigma; ADP was from P-L Biochemicals; ATP and oligomycin were from Boehringer; NADH and CCCP were from Calbiochem; EDTA and NaBr were from Mallinkrodt; Tris was from Schwarz/Mann; DCCD was from Aldrich; rotenone was from S. B. Penick; [^{32}P]phosphate was from ICN; [^{14}C]DCCD was from Research Products International. Oxonol VI and FSBA were gifts respectively from Dr. W. G. Hanstein, University of Bochum, and Dr. W. S. Allison, University of California, San Diego. Other reagents used were reagent grade or of the highest quality available.

Results

Properties of the F_1-Deficient Particles Used for Reconstitution. In order to assess properly the effect of partially inactivated F_1-ATPase by specific covalent modifiers on the kinetics of ATP synthesis and hydrolysis, it was necessary to obtain F_1-deficient particles with very low residual ATPase and oxidative phosphorylation activities that, however, were capable of combining with added, purified F_1 to reconstitute a system with high oxidative phosphorylation activity. Deficient particles prepared as described under Materials and Methods exhibited activities less than 1% of the ATPase of SMP and the oxidative phosphorylation of optimally reconstituted systems. This marginal ATP synthase activity was not increased upon addition of low levels of oligomycin, which indicated that the lack of ATP synthase activity was indeed due to F_1 deficiency rather than to oligomycin-repairable uncoupling that is seen in particles only partially depleted with respect to F_1. These highly depleted particles required both F_1 and OSCP for reconstitution of oxidative phosphorylation (Figures 1 and 2), and their ATP synthase activities were further increased upon addition of low levels of oligomycin (Figures 1 and 2). This activity increase was highest upon addition to the assay medium of about 0.2–0.4 μg of oligomycin/mg of particle protein (Figure 2B), and the oligomycin titer was the same for each preparation of F_1-deficient particles regardless of whether the reconstitution involved unmodified

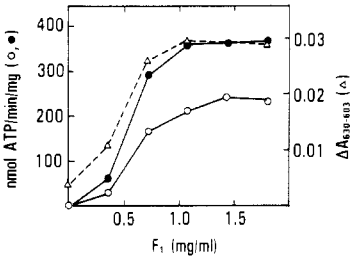

FIGURE 1: Effect of F_1 concentration on reconstitution of oxidative phosphorylation activity and membrane potential formation by F_1-deficient SMP. The reconstitution was carried out in a final volume of 100 μL of buffer as specified under Materials and Methods, containing 0.5 mg of ASU particles, 2.65 μg of OSCP, and the indicated amounts of F_1. Other conditions were as described under Materials and Methods. Where indicated (●), the assay mixture contained 0.21 μg of oligomycin/mg of particle protein.

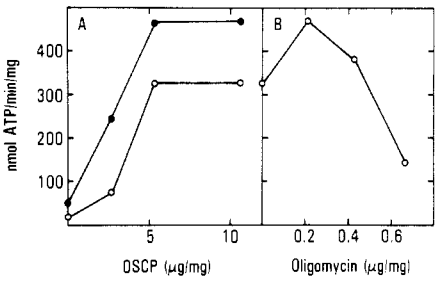

FIGURE 2: Effects of OSCP concentration in reconstitution (A) and of oligomycin concentration in the assay (B) mixtures of oxidative phosphorylation. The OSCP and oligomycin concentrations indicated are per milligram of particle protein. In (A), F_1 concentration in the reconstitution mixture was 0.34 mg/mg of ASU particles, and the filled circles indicate the presence of 0.21 μg of oligomycin/mg of particle protein in the assay mixtures. In (B), reconstitution was performed in the presence of 10.6 μg of OSCP/mg of particle protein. Other conditions were the same as in the open circles of panel A.

F_1 or F_1 treated with an inhibitor such as DCCD, FSBA, etc. This invariant optimal titer suggests that the proton leaks blocked by oligomycin could not be repaired by addition of F_1 plus OSCP and that partially inhibited F_1 was capable of reacting with the same number of F_1-deficient sites as unmodified F_1.

Under optimal conditions, the reconstituted particles were capable of ATP synthesis at the expense of succinate oxidation at rates in the range of 350–470 nmol min^{-1} (mg of protein)$^{-1}$ (Figures 1 and 2). Figure 1 also shows that the increase in ATP synthetic activity upon addition of F_1 to deficient particles plus OSCP was paralleled by the formation of respiration-induced membrane potential as monitored by the oxonol VI absorbance change at 630 minus 603 nm.

Modification of F_1 with DCCD. Incubation of F_1 with DCCD at 30 °C results in inhibition of ATPase activity and specific modification of the β-subunit (Pougeois et al., 1979; Yoshida et al., 1982). Studies in this laboratory (Wong et al., 1984) have shown that inhibition of the ATPase activity of F_1 up to about 90% is a linear function of DCCD binding and ≥90% inhibition corresponds to about 1.0 mol of DCCD bound/mol of F_1. Inhibition up to about 97% is associated with the binding of about 2 mol of DCCD/mol of F_1, while further DCCD binding (e.g., up to about 2.5 mol/mol of F_1) seems to increase the inhibition asymptotically toward 100%. This relationship between moles of DCCD bound and the degree of inhibition achieved is in agreement with catalytic cooperativity of the active sites (Gresser et al., 1982; Cross et al., 1982). Thus, inactivation of one site is associated with ~90% activity inhibition, modification of the second site increases the inhibition to ~97%, and the remaining residual

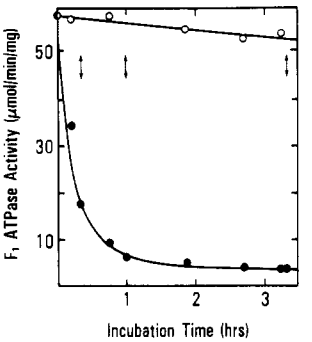

FIGURE 3: Modification of F_1 with DCCD. F_1 at 3.4 mg/mL was incubated at 30 °C with 0.2 mM DCCD (●) in a buffer containing 50 mM potassium phosphate, pH 6.8, 4 mM ATP, and 2 mM EDTA. At the times indicated by arrows, aliquots containing 0.27 mg of protein were removed, centrifuged through Sephadex G-25 columns equilibrated with a buffer containing 0.25 M sucrose, 50 mM Tris–acetate, pH 7.5, 2 mM ATP, and 2 mM EDTA, and reconstituted with F_1-deficient particles (0.77 mg of protein) in the presence of 16 μg of OSCP. The control F_1 not modified with DCCD (○) was treated and reconstituted in exactly the same manner. For the reconstituted activities, see Table I.

Table I: Activities of SMP Reconstituted with Unmodified and DCCD-Modified F_1-ATPase[a]

incubation time of F_1 (min)	oxidative phosphorylation[b]		oligomycin-sensitive ATPase[c]	
	control F_1	DCCD F_1	control F_1	DCCD F_1
20	211	110	4.38	1.14
60	201	73	4.33	0.60
200	155	55	3.50	0.18

	oxidative phosphorylation[b]	oligomycin-sensitive ATPase[c]
F_1-deficient SMP, no added F_1	<3	<0.07

[a] For experimental details, see Materials and Methods. Incubation of F_1 in the absence and presence of DCCD was carried out as in Figure 3. [b] nmol min^{-1} (mg of particle protein)$^{-1}$. [c] μmol min^{-1} (mg of particle protein)$^{-1}$.

activity is presumably due to uni-site ATP hydrolysis by F_1 (Wong et al., 1984).

In our studies, purified F_1 was incubated with DCCD until ≥90% ATPase activity inhibition was obtained. Analyses with [^{14}C]DCCD showed that, as in the studies described above, this degree of inhibition corresponded to ≥1.0 mol of DCCD bound/mol of F_1. At several points during the course of incubation of F_1 with DCCD, as shown in Figure 3, samples were removed, freed of excess DCCD by passage through Sephadex columns as described, assayed to determine the degree of ATPase inhibition, and used for reconstitution with F_1-depleted particles plus OSCP. Table I shows the data for oxidative phosphorylation and oligomycin-sensitive ATPase activities of systems reconstituted with DCCD-treated and control F_1 samples incubated at 30 °C for the periods of time shown by arrows in Figure 3. In this figure, the arrows at 20, 60, and 200 min of incubation indicate, respectively, about 70, 87, and 93% inhibition of F_1 by DCCD. In the reconstituted system the corresponding degrees of inhibition of oligomycin-sensitive ATPase were 74, 86, and 95%. By comparison, however, the inhibition of the oxidative phosphorylation activities of the DCCD-treated samples were, respectively, 48, 64, and 65%. Of particular interest is the oxidative phosphorylation activity of the sample whose ATPase activity was >90% inhibited by prolonged incubation with DCCD. This sample contained ≥1 mol of DCCD/mol of F_1, i.e., an average

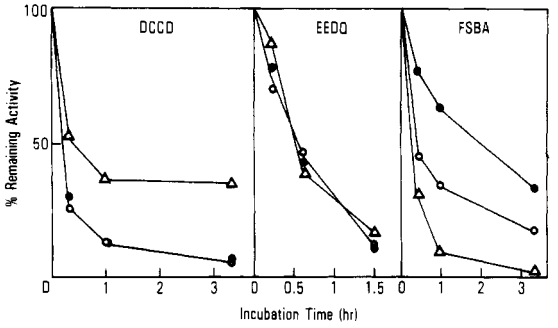

FIGURE 4: Activities of F_1-deficient particles reconstituted with F_1 modified with DCCD (left panel), EEDQ (center panel), or FSBA (right panel). F_1 modifications were carried out as in Figure 3, except that in the case of EEDQ and FSBA the incubation buffer contained 40 mM Tris–sulfate, pH 7.0, 1 mM EDTA, and where indicated 0.6 mM EEDQ or 2.0 mM FSBA. Activities are shown as percent of the ATPase activity of unmodified F_1 before reconstitution (●) and of the oligomycin-sensitive ATPase (○) and oxidative phosphorylation (△) activities of particles reconstituted with unmodified F_1. The latter two control activities at zero time were for the DCCD, the EEDQ, and the FSBA experiments respectively 4.5, 8.9, and 8.5 μmol min^{-1} mg^{-1} (○) and 220, 300, and 264 nmol min^{-1} mg^{-1} (△).

of one inactivated β-subunit/mol of F_1. Yet it was capable of oxidative phosphorylation at an appreciable rate (note in Table I that both the ATPase and oxidative phosphorylation activities of the deficient particles in the absence of added F_1 were negligible). Unless we assume a mixed population of DCCD-modified and unmodified F_1 molecules and preferential reconstitution of the latter, the results of Table I would suggest that three active β-subunits per F_1 may not be obligatory for ATP synthesis, just as the findings of Grubmeyer et al. (1982) and Cross et al. (1982) indicate that functionality of all three sites is not necessary for ATP hydrolysis [see also Steinmeier & Wang (1979) and Kohlbrenner & Boyer (1982)]. We do not think that the possibility of preferential binding of unmodified F_1 is very likely because, with the exception of FSBA, the degree of inhibition of oligomycin-sensitive ATPase after reconstitution was always the same as the extent of inhibition of the inhibitor-treated F_1 before reconstitution. In addition, the systems reconstituted with modified F_1 exhibited an apparent $K_m^{P_i}$ in oxidative phosphorylation that was twice that of the control particles reconstituted with unmodified F_1 (see below).

Modification of F_1 with Other Inhibitors. In addition to DCCD, six other inhibitors were tested, most of which are considered to react covalently with the β-subunit. These inhibitors were FSBA (Esch & Allison, 1978), EEDQ (Ting & Wang, 1980; Satre et al., 1983), FDNB (Andrews & Allison, 1981), CMCD (Kozlov & Skulachev, 1977), QM (Laikind & Allison, 1983), and dansyl-Cl (this work). In each case, F_1 modification, reconstitution, and assays were similar to those described above for DCCD. Typical results for DCCD, EEDQ, and FSBA are summarized in Figure 4. In the case of DCCD, similar degrees of inhibition were obtained as a function of incubation time for the ATPase activity of F_1 before and after reconstitution, the latter being >95% oligomycin-sensitive. However, as described above, there was much less inhibition of oxidative phosphorylation. With EEDQ, all three activities, i.e., the ATPase activity of F_1, the oligomycin-sensitive ATPase activity of reconstituted particles, and the oxidative phosphorylation activity of the reconstituted particles, were inhibited to the same extent. With FSBA, the picture was very different. The relative extents of inhibition of the three activities at each incubation time point were oxidative phosphorylation > oligomycin-sensitive ATPase of particles > ATPase of F_1. The other inhibitors mentioned

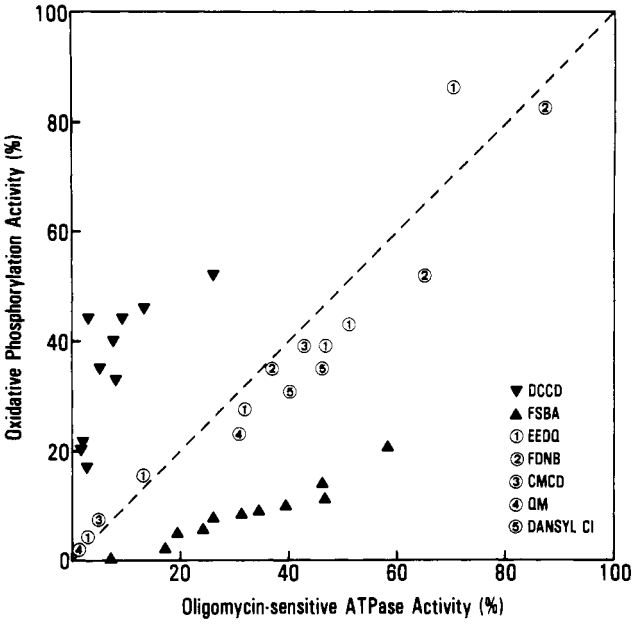

FIGURE 5: Correlation between the oxidative phosphorylation and the oligomycin-sensitive ATPase activities (percents relative to controls) of particles reconstituted with F_1 that had been inhibited to various extents by modification with the reagents shown. The experimental conditions were the same as described in the legends to Figures 3 and 4. The dashed line represents the same degree of inhibition of the rates of ATP synthesis and hydrolysis.

above behaved like EEDQ, showing essentially the same degree of inhibition for oxidative phosphorylation and oligomycin-sensitive ATPase activity of the reconstituted particles. Figure 5 summarizes these comparative results. It is seen that the data for EEDQ, FDNB, CMCD, QM, and dansyl-Cl fall near the theoretical line for the same degree of inhibition of oxidative phosphorylation and oligomycin-sensitive ATPase activities. However, DCCD modification of F_1 inhibited ATP hydrolysis much more than synthesis, while FSBA modification of F_1 had the opposite effect, inhibiting ATP synthesis much more than hydrolysis.

The unusual behavior of DCCD- and FSBA-modified F_1 in ATP synthesis and hydrolysis made it necessary to examine other properties of the system. For example, it was possible that FSBA modification of F_1 might have caused uncoupling as does the modification of chloroplast F_1 with the bifunctional maleimide *o*-phenylenedimaleimide (Weiss & McCarty, 1977). However, as seen in Figure 6A, respiration-induced membrane potential was the same in particles reconstituted with unmodified F_1 and three preparations of DCCD-modified F_1 in which ATPase activity was inhibited by 91, 97, and >98%. The small decrease in the extent of oxonol VI absorbance change in going from 91 to >98% inhibition was the result of prolonged incubation of F_1 at 30 °C and was essentially the same for both the unmodified and DCCD-treated F_1. Similar results are given in Figure 6B for FSBA-modified F_1 (as well as all the other inhibitors used, data not shown). Again, the respiration-induced oxonol VI absorbance change was the same in particles reconstituted with unmodified and FSBA-modified F_1 in the absence and presence of oligomycin, even though the particles containing FSBA-treated F_1 were inhibited by 94% relative to the control in oxidative phosphorylation activity. The bottom trace in this figure shows that the F_1-deficient ASU particles were uncoupled and could not build up a membrane potential, a defect that was partially corrected by reconstitution with either F_1 or FSBA-modified F_1 and much more fully after treatment with low levels of oligomycin (Figure 6B).

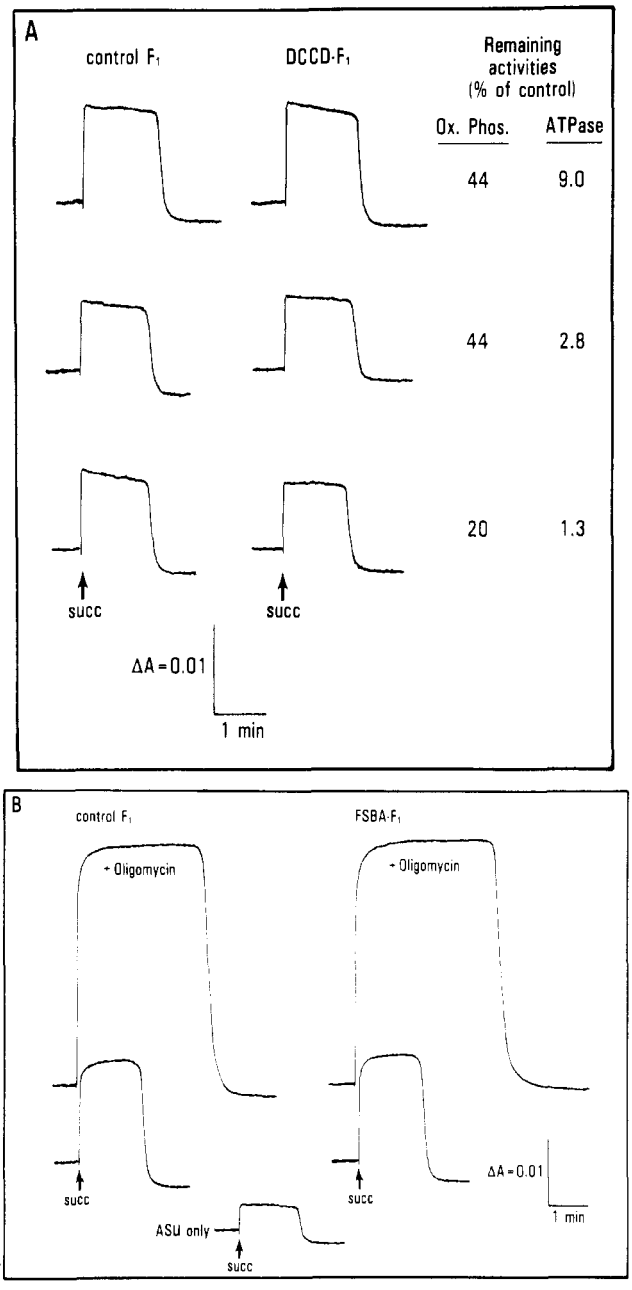

FIGURE 6: Formation of membrane potential induced by succinate oxidation in F_1-deficient particles reconstituted with DCCD-treated (A) or FSBA-treated (B) F_1. Membrane potential formation was monitored at 30 °C by the absorbance change of oxonol VI. In (A), F_1 was incubated in the absence and presence of 0.2 mM DCCD for 60 (upper traces), 180 (middle traces), and 360 min (lower traces) before reconstitution. The oxidative phosphorylation and oligomycin-sensitive ATPase activities of reconstituted systems containing DCCD-treated F_1 are shown as percent of the activities of the corresponding control particles reconstituted with F_1 incubated in the absence of DCCD. In (B), F_1 was incubated in the absence and presence of 2 mM FSBA for 77 min. The oxidative phosphorylation and oligomycin-sensitive ATPase activities of the particles reconstituted with FSBA-treated F_1 were respectively 5.8 and 24% of the control. In the presence of low levels of oligomycin (0.4 μg/mg of particle protein) the static-head membrane potential and the oxidative phosphorylation activity of the particles were considerably increased. The bottom trace in (B) shows the extent of membrane potential formed by F_1-deficient ASU particles plus OSCP before reconstitution with F_1.

It was also important to check in particles reconstituted with F_1 modified with DCCD, FSBA, or EEDQ the K_m values for ADP, P_i, and ATP, respectively, in oxidative phosphorylation and oligomycin-sensitive ATPase assays. These results are shown in Figures 7 and 8. As seen in Figure 7, the apparent

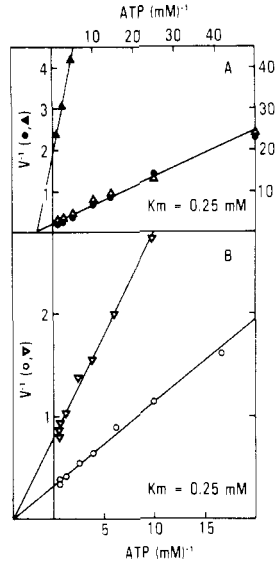

FIGURE 7: Double-reciprocal plots showing the effect of F_1 modification with DCCD (▲ and △) (panel A), EEDQ (▽) (panel B), or FSBA (○) (panel B) on the apparent K_m^{ATP} in oligomycin-sensitive ATPase assays of the reconstituted particles. Open triangles (△) in panel A show the data for DCCD plotted against the contracted right ordinate. Where indicated, F_1 at 3.4 mg/mL was treated at room temperature with 0.6 mM DCCD for 66 min and 0.5 mM FSBA or 0.6 mM EEDQ, both for 30 min, and was reconstituted with ASU particles as described under Materials and Methods. Filled circles (●) denote particles reconstituted with unmodified F_1. The reconstituted particles were washed once by suspension in cold 0.25 M sucrose containing 10 mM Tris–acetate, pH 7.5, and centrifugation at 5000g for 3 min. After a washing, the oligomycin sensitivity of the ATPase activity of the reconstituted particles was >95%, regardless of the nature of the F_1 modifier used.

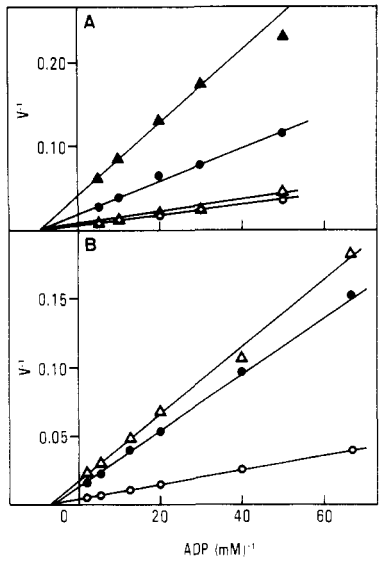

FIGURE 8: Double-reciprocal plots showing the effect of F_1 modification with DCCD (● and ▲) (panel A), FSBA (△) (panels A and B), or EEDQ (●) (panel B) on the apparent K_m^{ADP} in oxidative phosphorylation assays of the reconstituted particles. Where indicated, F_1 was treated at 30 °C with 0.3 mM DCCD for 64 min (●) or 0.45 mM DCCD for 190 min (▲). Concentrations of F_1, FSBA, and EEDQ and incubation conditions for FSBA and EEDQ were the same as those in Figure 7. Open circles (○), particles reconstituted with unmodified F_1. The extents of inhibition of the ATPase activity of F_1 after treatment with the above inhibitors were as follows: DCCD 1 (●), 94%; DCCD 2 (▲), 99%; FSBA, 17%; EEDQ, 70%. The reconstitutions were performed in the absence of added ATP as described under Materials and Methods.

K_m^{ATP} was unchanged for the oligomycin-sensitive ATPase activities of particles reconstituted with unmodified F_1 or with

F_1 that had been modified with FSBA, EEDQ, or DCCD, resulting in 41, 76, and 90% inhibition of ATPase activity, respectively. This K_m (250 μM) is also essentially the same as the apparent K_m^{ATP} of "intact" SMP. Figure 8 shows the apparent K_m^{ADP} in oxidative phosphorylation assays with particles reconstituted with unmodified F_1 and DCCD- (two levels of inhibition, Figure 8, top panel), FSBA-, or EEDQ-modified F_1 (Figure 8, bottom panel). Once again, it is seen that the apparent K_m^{ADP} values (110–150 μM) are the same for particles containing unmodified and inhibitor-modified F_1. While essentially unchanged in the reconstituted systems, these apparent K_m^{ADP} values are considerably higher, however, than the apparent K_m^{ADP} of intact SMP ($\sim$30 μM) (Hatefi et al., 1982). The apparent $K_m^{P_i}$ in oxidative phosphorylation catalyzed by reconstituted particles was also determined (data not shown). The particles reconstituted with unmodified F_1 exhibited an apparent $K_m^{P_i}$ value (0.79 mM) very close to that of intact SMP (0.65 mM) (Hatefi et al., 1982). However, the particles containing FSBA-, EEDQ-, or DCCD-modified F_1 exhibited much higher apparent $K_m^{P_i}$ values than the control, but again as in the case of K_m^{ATP} and K_m^{ADP} (Figures 7 and 8), the particles containing different kinds of modified F_1 showed the same apparent $K_m^{P_i}$ (1.6 mM).

Discussion

Recent evidence has indicated that the individual active sites on the three β-subunits of F_1-ATPase are catalytically active in ATP hydrolysis and interact in a manner that results in (a) negative cooperativity with respect to ATP concentration and (b) positive catalytic cooperativity in the sense that substrate binding to the second and third sites greatly facilitates turnover at the first site (Cross et al., 1982; Gresser et al., 1982). While it is not known whether the individual active sites are each capable of ATP synthesis, the results of Hackney & Boyer (1978) and Nalin & Cross (1982) have suggested to these authors a requirement in ATP synthesis for at least two cooperatively interacting catalytic sites.

In the present study, we modified isolated F_1-ATPase with inhibitors known to bind covalently and in most instances specifically to the β-subunit (FSBA reacts with both the α- and β-subunits). The inhibitors were selected with regard to their covalent, stable interaction, preferably with the β-subunit, and for lack of evidence regarding migration from one site to another. For this reason, Nbf-Cl, which is known to migrate from a tyrosyl to an amino group and is easily removable, was not employed. Also, the procedures for removal of F_1 (+OSCP) from SMP and for reconstitution of the denuded particles with added F_1(+OSCP) were perfected to the point that the F_1-deficient particles had retained less than 1% ATPase or oxidative phosphorylation activity, even in the presence of low levels of oligomycin, and that after addition of unmodified F_1 plus OSCP they exhibited reconstituted oxidative phosphorylation activities as high as 500 nmol min^{-1} (mg of particle protein)$^{-1}$ in the presence of succinate as the oxidizable substrate. The results of reconstitution in experiments in which chemically modified F_1 was used showed the following.

(1) Except for DCCD and FSBA, F_1 modification by other reagents (EEDQ, FDNB, CMCD, QM, dansyl-Cl) resulted in the same degree of inhibition of ATPase activity before reconstitution as of oligomycin-sensitive ATPase and oxidative phosphorylation activities after reconstitution (Figures 4 and 5). A simple interpretation of these results is the nonselective binding to deficient particles of unmodified and partially (one or two sites inactivated) or fully (all three sites inactivated) modified F_1, thus resulting in the same degree of inhibition of the reconstituted system as compared to the inhibitor-treated F_1 before reconstitution. Another possibility is that the inhibitors caused complete inhibition of a fraction of F_1 molecules and that the activities experienced before and after reconstitution were representative of the unmodified F_1 molecules. This second possibility is not in agreement with the mode of inhibition of several F_1 inhibitors. For example, we have shown with radioactive DCCD, Nbf-Cl (Wong et al., 1984), and the ATPase inhibitor protein (Wong et al., 1982), all of which bind only to the β-subunit, that up to 1 mol of inhibitor/mol of F_1, inhibitor binding is linearly related to activity inhibition, the latter proceeding to 90–95%. Additional inhibitor binding and further inhibition are relatively very slow, and indeed, it is extremely difficult to reach the saturation point of 3 mol of inhibitor/mol of F_1. This behavior of the enzyme toward inhibitors, which is analogous to its negative cooperativity with respect to ATP, makes the possibility of complete inhibition of a fraction of F_1 molecules much less likely than the first possibility discussed above. This conclusion also agrees with the effect of F_1 modification on the apparent $K_m^{P_i}$ in oxidative phosphorylation assays (see above). Indeed, since in most instances in the data shown in Figure 5 the degree of inhibition of ATPase activity was <90%, it is highly likely that the modifications involved only one β-subunit per molecule of F_1. In such a case, the data of Figures 4 and 5 for EEDQ, FDNB, CMCD, QM, and dansyl-Cl would suggest an important point, namely, that the degree of catalytic cooperativity as affected by inhibition of one active site per F_1 is essentially the same for the ATPase activity of isolated F_1 and the ATP synthetic and hydrolytic activities of membrane-bound F_1.

(2) In the case of FSBA, the relative extents of inhibition were oxidative phosphorylation activity of reconstituted SMP > oligomycin-sensitive ATPase activity of reconstituted SMP > ATPase activity of F_1 (Figure 4). These results are difficult to rationalize, especially since in our hands [^{14}C]FSBA was found to bind to both the α- and the β-subunits and the α-subunit has been suggested by others to participate in catalysis (Lunardi & Vignais, 1982; Williams & Coleman, 1982; Senior & Wise, 1983).

(3) In contrast to FSBA, DCCD up to about 2.5 mol/mol of F_1 was found to bind only to the β-subunit [regarding the possible differences in the inhibitory characteristics of DCCD and EEDQ, see Pougeois (1983)], and the data for DCCD-modified F_1 showed much greater inhibition of ATP hydrolysis than synthesis (Figures 4 and 5). In one experiment, the inhibition of ATPase activity was carried to 99%, and [^{14}C]DCCD binding data indicated the incorporation of $\geq$2 mol of DCCD/mol of F_1. Yet this reconstituted system exhibited an oxidative phosphorylation activity that was 20% of the control. These results can be explained, as others have done with the use of other inhibitors (Steinmeier & Wang, 1979; Kumar et al., 1979; Schafer, 1982), in terms of different mechanistic paths for ATP synthesis and hydrolysis that are inhibited to different extents by DCCD. Another possible explanation, which circumvents the unlikely assumption of separate paths for ATP synthesis and hydrolysis, is that DCCD modification exerts a greater effect on the catalytic cooperativity of ATP hydrolysis than that of ATP synthesis. A third possibility is that DCCD inhibits by interfering with substrate binding, but it interferes more with ATP binding than with the binding of ADP and P_i (see, however, point 4).

(4) In spite of the different inhibitory effects of DCCD, FSBA, and the other five inhibitors examined (Figure 5), the apparent K_m values of the modified and reconstituted F_1 preparations did not show any differences. For F_1 preparations modified to different extents by DCCD, FSBA, and EEDQ,

the apparent K_m values for ATP in ATP hydrolysis and for ADP and P_i in ATP synthesis were in each case the same regardless of the nature of the modifier or the degree of inhibition. The only differences observed were that (a) the apparent K_m^{ADP} values of the reconstituted particles (including the control that was reconstituted with unmodified F_1) were 4–5 times that of intact SMP and (b) the apparent $K_m^{P_i}$ values of the reconstituted particles containing modified F_1 were twice that of the particles reconstituted with unmodified F_1. Once again, we have no satisfactory explanation for these results, especially since what is known about the binding sites and the mechanism of action of these inhibitors is too meager to help in rationalizing the above data. Only FSBA is considered to be an analogue of ATP and is known to bind to a tyrosyl residue, presumably at the active site of the β-subunit (Esch & Allison, 1978). However, it should be pointed out that these apparent K_m values as determined from double-reciprocal plots are not necessarily very precise. This is because for assay of ATPase activity the reconstituted particles are not as stable as SMP and the substrate ranges that could be used with the inhibited systems could not be wide enough to allow detection of possible slope changes in these plots at very low substrate concentrations.

Regardless of the above peculiarities of the inhibitors examined and the complexity of the results, one point is relatively clear: F_1 containing per mole at least 1 mol of DCCD covalently linked to the β-subunit(s) and inhibited up to 99% for ATP hydrolysis is still capable of ATP synthesis at an appreciable rate when recombined with F_1-free SMP. This finding is supported by data presented above for other inhibitors (except FSBA) and agrees with the results of Steinmeier & Wang (1979) and Kohlbrenner & Boyer (1982) on the ATP synthetic activity of systems reconstituted with Nbf-modified F_1. It is also consistent with the findings of Grubmeyer et al. (1982) and Cross et al. (1982), who showed that F_1-ATPase can function without the participation of all three active sites in catalysis.

Acknowledgments

We thank Dr. T. Yagi and S.-Y. Wong for help and advice and C. Munoz for the preparation of mitochondria.

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