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# Reconstitution of Oxidative Phosphorylation by Chemically Modified Coupling Factor $F_1$: Differential Inhibition of Reactions Catalyzed by $F_1$ Labeled with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole or 2,3-Butanedione†

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ABSTRACT: Energy coupling factor $F_1$ from beef heart mitochondria has been chemically modified by either 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) or 2,3-butanedione. Labeled $F_1$ was used for the reconstitution of oxidative phosphorylation in urea-washed submitochondrial particles (ASU particles). Inhibition of ATPase activity by NBD-Cl follows a simple exponential decay, reaction is first order with respect to NBD-Cl, and magnesium complexes of methylene analogues of ADP or ATP produce sevenfold reduction in the rate of inhibition. Spectral evidence indicates labeling of tyrosine, with a biphasic incorporation approaching 2 mol of NBD label per mol of $F_1$. Experiments on $F_1$ involving labeling by both NBD-Cl and 2,3-butanedione reveal no competition between the two labeling agents. When ASU particles are reconstituted with $F_1$ containing 1.35 to 1.65 mol of NBD label/mol of $F_1$, the activities for ATP $\rightleftharpoons$ $P_i$ exchange, ATP-driven reverse electron transport, and membrane-bound ATPase are almost completely inhibited. However, the remaining activity for net synthesis of ATP is 35–65% of the initial value. For ASU particles reconstituted with 2,3-butanedione labeled $F_1$, the loss of activity for reverse electron transport occurs at a fivefold greater rate than loss of activity for net ATP synthesis, emphasizing a functional separation of these processes. These results are difficult to rationalize by a compulsory alternating site model but can be explained by the presence of catalytic sites specialized for ATP utilization and synthesis, respectively.

Coupling factor $F_1$, first isolated by Racker and co-workers (Pullman et al., 1960; Penefsky et al., 1960), is generally regarded as the terminal enzyme of oxidative phosphorylation. Its known properties have been recently reviewed and emphasis has been placed on the remarkable complexity of this enzyme (Pedersen, 1975; Panet & Sanadi, 1976). Studies by Senior (1975) and Wagenvoord et al. (1977) support a subunit stoichiometry of $\alpha_2\beta_2\gamma_2\delta_x\epsilon_2$, where $x$ is presumably 1 or 2. Preliminary crystallographic studies have revealed a twofold axis of symmetry (Amzel & Pedersen, 1978).

It is clear from work in several laboratories that $F_1$ possesses several nucleotide binding sites, including 2 sites for tightly bound ADP and 0, 1, or 3 sites for tightly bound ATP

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(Leimgruber & Senior, 1976a; Garrett & Penefsky, 1975; Rosing et al., 1975). However, neither the location of these sites nor their function in oxidative phosphorylation is clear. Numerous active site studies on $F_1$ have been concerned with inhibition of the ATPase reaction catalyzed by purified $F_1$ and, to a lesser extent, by membrane-bound $F_1$. In addition, a growing body of evidence has shown that some substances which inhibit ATPase activity do not produce equivalent inhibition of net ATP synthesis, ATP-driven reverse electron transport, energy-linked nicotinamide nucleotide transhydrogenase activity, and/or various exchange reactions. For example, studies using the native ATPase inhibitor protein (Ernster et al., 1973), quercetin (Lang & Racker, 1974), and AMP-PNP[1] (Penefsky, 1974; Pedersen, 1975b) have demonstrated that, under conditions where these substances strongly inhibit ATPase activity and ATP-driven reverse electron transport, they have relatively little effect on net ATP synthesis. AMP-PNP also inhibits the ATP $\rightleftharpoons$ $P_i$ and $P_i$ $\rightleftharpoons$ $H_2O$ exchanges but not the ATP $\rightleftharpoons$ $H_2O$ exchange (Holland et al., 1974). On the other hand, use of the inhibitors aurovertin (Lee & Ernster, 1968) or spegazzinine (Roveri & Vallejos, 1974) or low level trypsin treatment (Leimgruber & Senior, 1976a) all produce much greater effects on net ATP synthesis than on ATPase activity.

Such differential effects of inhibitors have led to the suggestion that separate sites on mitochondrial $F_1$ may be specialized for ATP synthesis and ATP utilization, respectively (Penefsky, 1974; Pedersen, 1975b). Based on various kinetic studies, Lardy and co-workers have proposed that ATP synthesis and utilization both take place at a catalytic site but that a separate nucleotide binding site functions as a regulatory site (Lardy et al., 1975; Schuster et al., 1975). Clearly, to distinguish between such models and to achieve a more detailed understanding of the terminal steps in oxidative phosphorylation, it is necessary to obtain further information about the nature and function of sites on $F_1$ involved in catalysis or regulation. To this end, we have employed NBD-Cl and 2,3-butanedione, which were previously shown to inactivate ATPase activity in isolated $F_1$ by chemical modification of specific tyrosine (Ferguson et al., 1975) and arginine (Marcus et al., 1976) residues, respectively. Using ASU particles (Racker & Horstman, 1967) reconstituted with OSCP and modified $F_1$, we have examined the effects of specific chemical modification on various catalytic activities associated with membrane-bound $F_1$.

## Experimental Procedures

*Materials.* Pyruvate kinase (type II), L-lactic dehydrogenase (type III), hexokinase (type IV), bovine serum albumin, dithiothreitol, phosphoenolpyruvate, ATP (equine muscle), ADP (grade I), IDP, NADH (grade III), $NAD^+$, antimycin A, oligomycin, and NBD-Cl were purchased from Sigma Chemical Co. 2,3-Butanedione was purchased from Aldrich Chemical Co. Phosphorus-32 in the form of orthophosphoric acid was purchased from New England Nuclear. Sephadex G-25, DEAE-Sephadex A-50, and CM-Sephadex C-25 used in protein preparations were products of Pharmacia Fine Chemicals, Inc. Ammonium sulfate and urea employed in protein preparations were of UltraPure grade from Schwarz/Mann, Orangeburg, N.Y. Poly(ethylenimine), 50% aqueous, obtained from Eastman, Rochester, N.Y., was used in the preparation of PEI paper (Randerath, 1963). Other chemicals used were of reagent grade and were used without further purification.

*Protein Preparation.* The starting material for all protein preparations was bovine heart mitochondria, prepared essentially as described by Löw & Vallin (1963). Coupling factor $F_1$ was prepared by the method of Knowles & Penefsky (1972). Different batches of purified $F_1$ had specific activities in the range 70 to 80 units/mg. On polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and mercaptoethanol (Weber & Osborn, 1969), purified $F_1$ yielded five bands, of approximate molecular weights: 56 000; 51 000; 33 000; 14 000; and 8000. $F_1$ was stored at +5 °C as a precipitate in buffer containing 0.25 M sucrose, 50 mM Tris-Cl, pH 8.0, 2 mM EDTA, 4 mM ATP, and 2 M ammonium sulfate. Oligomycin sensitivity conferring protein (OSCP) was purified to homogeneity by the method of Senior (1971). It was stored at +5 °C as a precipitate in medium containing 20 mM Tris·sulfate, pH 8.0, and 2.8 M ammonium sulfate. A particles were prepared as previously described (Higashiyama et al., 1975). Sonications were performed by a Branson W-350 sonifier with a $^1/_2$-in. step horn. ASU particles were prepared from A particles exactly as described by Racker & Horstman (1967), except that the final resuspension medium did not contain DTT. The presence of DTT was not required for ASU particle activity and added DTT did not stimulate activity. ASU particles were stored in 0.5-mL aliquots, 20 mg/mL protein concentration, at −70 °C. ETPH's ($Mg^{2+}$, $Mn^{2+}$) were prepared according to Beyer (1967).

*Reconstitution of ASU Particles.* Reconstitution was routinely performed at 30 °C in buffer containing 50 mM triethanolamine hydrochloride, 0.2 M sucrose, 2 mM ATP, and 2 mM EDTA, pH 7.5. In experiments utilizing $F_1$ modified with 2,3-butanedione, the reconstitution medium also contained 0.1 M borate and the pH was 8.0. Reconstitutions utilized 20 μg of OSCP and 300 μg of $F_1$ (either native or chemically modified $F_1$) per mg of ASU particle protein. ASU particles were incubated 1 min with OSCP only and then an additional 3 min following addition of the $F_1$ sample. The final concentration of ASU particle protein in the mixture was 5 mg/mL. Reconstituted ASU particles were stored on ice during the course of the experiments, which were usually completed within 1 to 2 h following the reconstitution. It was also found that particles reconstituted with native $F_1$ or NBD-$F_1$ survive freezing and storage at −70 °C without loss of the assayed activities.

*Assays.* ATPase assays were carried out at 30 °C in a total volume of 3.0 mL containing 50 mM Tris·acetate, 3 mM $MgCl_2$, 10 mM KCl, 2 mM ATP, 2 mM PEP, 0.2 mM NADH, 100 μg of pyruvate kinase, and 40 μg of L-lactic dehydrogenase, pH 7.5. Absorbance changes at 340 nm were followed in a Gilford Model 2000 spectrophotometer. Assays of ATP-driven reverse electron transport were performed at 30 °C in a total volume of 3.0 mL containing 50 mM Tris·sulfate, 3.3 mM $MgCl_2$, 10 mM KCl, 6.7 mM succinate, 2 mg of BSA, 2 mM ATP, 1 mM $NAD^+$, 10 mM PEP, 100 μg of pyruvate kinase, and 3.3 mM KCN, pH 7.8. Reaction was

[1] Abbreviations used: AMP-PNP, adenylyl imidodiphosphate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; $P_i$, inorganic phosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; OSCP, oligomycin sensitivity conferring protein; ASU particles, submitochondrial particles prepared from beef heart mitochondria by sonication in the presence of ammonium hydroxide at pH 9 followed by steps involving Sephadex and urea treatments; NBD-$F_1$, coupling factor $F_1$ labeled by NBD-Cl; $F_1$·ASU and NBD-$F_1$·ASU, ASU particles reconstituted with OSCP plus $F_1$ and NBD-$F_1$, respectively; PEI, poly(ethylenimine); DEAE, diethylaminoethyl; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; PEP, phosphoenolpyruvate; BSA, bovine serum albumin.

initiated by addition of the cyanide. Reconstituted ASU particles possess appreciable ATPase activity, which necessitates the presence of an ATP-regenerating system to obtain linear reaction traces at 340 nm.

Measurements of oxidative phosphorylation utilized a system containing 50 mM triethanolamine hydrochloride, 0.1 M sucrose, 0.5 mM EDTA, 50 mM glucose, 27 units of hexokinase, 1 mg of BSA, 20 mM $MgCl_2$, 10 mM ADP, 30 mM succinate, and 20 mM potassium phosphate containing sufficient $^{32}P_i$ to produce a specific activity of about 0.15 mCi/mol. Other conditions were pH 7.5, 30 °C, and 1.0-mL total volume. Assays routinely used 0.25 mg of reconstituted ASU particles and a 3-min fixed time period. Reactions were quenched by adding perchloric acid to a final concentration of 3.5%. Net ATP synthesis was determined from the formation of phosphorus-32-labeled glucose 6-phosphate. The analysis was performed by spotting (centrally in a 3-cm wide channel) an aliquot of each acidified reaction mixture on Whatman no. 541 chromatography paper, drying, and developing overnight with solvent 1 as described by Wood (1968). After drying, chromatography papers were ruled in 1-cm increments and cut into 1 × 3 cm strips, which were counted in a Beckman LS-233 scintillation counter. With solvent 1, glucose 6-phosphate migrates faster than ATP + ADP but slower than AMP and orthophosphate, allowing a clear-cut evaluation of radioactivity present in glucose 6-phosphate. In a few phosphorylation experiments the hexokinase–glucose trap was omitted from the reaction mixture, and ATP synthesis was directly evaluated from the incorporation of $^{32}P_i$ into ATP. For this purpose, adenine nucleotides were separated on PEI paper prepared according to Randerath (1963). The developing solvent was 0.25 M Tris–acetate, pH 4.7, containing 1.0 M NaCl. Developing for about 5 h at room temperature gave complete separation of ATP, ADP, and AMP. Orthophosphate and AMP were not completely separated.

Assays of the ATP $\rightleftharpoons$ $^{32}P_i$ exchange reaction were carried out at pH 7.5, 30 °C, in a total volume of 1.0 mL containing 50 mM triethanolamine hydrochloride, 0.1 M sucrose, 0.5 mM EDTA, 1 mg of BSA, 4 μM rotenone, 4 μM antimycin, 20 mM $MgCl_2$, 20 mM ATP, and 20 mM potassium phosphate containing sufficient $^{32}P_i$ to produce a specific activity of about 0.15 mCi/mmol. The reaction was initiated by adding a mixture containing the phosphate and ATP–Mg and was terminated after a 3-min incubation by adding perchloric acid to a final concentration of 3.5%. The amount of radioactive ATP formed was evaluated by chromatography on PEI paper as described above.

Concentrations of ADP (or ATP) and IDP were determined from the absorbance at 259 and 248.5 nm, assuming millimolar extinction coefficients of 15.4 and 12.2, respectively (Beaven et al., 1955).

*Respiration Measurements.* Rates of oxygen consumption by reconstituted ASU particles were measured using a standard Clark type polarographic electrode (YSI 5331). The buffer medium was identical with that used in the assay of ATP synthesis, except that hexokinase, BSA, and ADP were omitted. For evaluation of P:O ratios, respiratory control ratios, and stimulation by uncoupler, respiration measurements were performed within 1 h following reconstitution of the ASU particles. The sample volume was 3.0 mL and the temperature was 30 °C, unless specified otherwise in the text.

*Chemical Modification of $F_1$.* Preparations of $F_1$ were stored at +5 °C as an ammonium sulfate precipitate (25–50 mg/mL of total protein), as described previously. Just prior to use, an aliquot of the $F_1$ suspension was centrifuged and the pellet was redissolved in the appropriate buffer to a protein concentration of approximately 10 mg/mL. For modification by NBD-Cl, the buffer contained 50 mM triethanolamine hydrochloride, 0.2 M sucrose, 4 mM ATP, and 4 mM EDTA, pH 7.5. The solution of $F_1$ in this buffer was divided into two portions. To one portion was added sufficient 20 mM NBD-Cl (in ethanol) to attain a final concentration of 100 μM NBD-Cl in the reaction mixture. To the second portion, the control, was added an equivalent volume of ethanol. After a 2-h incubation at 25 °C in the dark, both samples were applied to separate columns of Sephadex G-25 fine, 1 × 20 cm, equilibrated with the above triethanolamine buffer. Fractions containing $F_1$ were identified by assay of their ATPase activity. Fractions containing NBD-$F_1$ were assayed for ATPase in the presence of DTT to remove the NBD label. After pooling appropriate fractions, protein concentrations of the two samples were measured by biuret using a BSA standard. Since triethanolamine interferes with the biuret color development, protein samples were precipitated with 4% trichloroacetic acid, centrifuged, and redissolved in 0.5 M NaOH before addition of the biuret reagent. When prepared as described above, the ATPase activity of NBD-$F_1$ was at least 97% inhibited compared with the control. Incorporation of the NBD label was also monitored by following the absorbance increase of a new peak at 390 nm, as observed by Ferguson et al. (1975). Absorbance scans from 350 to 500 nm employed an Aminco DW-2 spectrophotometer. The $F_1$ and NBD-$F_1$ samples could be kept for at least 24 h without significant changes in properties.

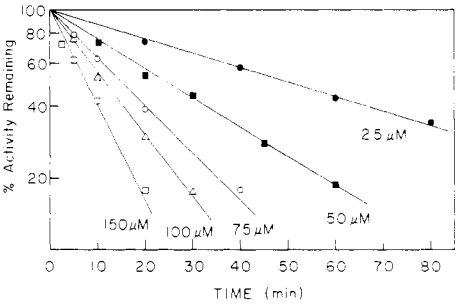


FIGURE 1: Inactivation of ATPase activity by NBD-Cl. The time course of inactivation is shown for different concentrations of NBD-Cl, as indicated in the figure. Reaction conditions were: 50 mM triethanolamine hydrochloride, 0.2 M sucrose, 4 mM ATP, 4 mM EDTA, pH 7.5, 25 °C, and 2 μM $F_1$.

Chemical modification by 2,3-butanedione was performed under conditions generally similar to those described for NBD-Cl. However, the buffer system was 50 mM triethanolamine hydrochloride, 0.2 M sucrose, 4 mM ATP, 4 mM EDTA, and 0.1 M borate, pH 8.0. Final concentrations of 2,3-butanedione in the reaction mixture ranged from 2 to 5 mM and incubations were at 25 °C. Excess reagent was removed from the samples by desalting on columns of Sephadex G-25. As many as seven different reaction times with 2,3-butanedione were examined in a given experiment. Protein samples were used within 24 h of preparation.

## Results

*Reaction of NBD-Cl with $F_1$.* The ATPase activity of coupling factor $F_1$ from beef heart mitochondria is inactivated by NBD-Cl as a result of specific modification of approximately one tyrosine residue per enzyme molecule (Ferguson et al., 1975). We have further characterized the effects of the NBD label both on soluble $F_1$ and on $F_1$ rebound to vesicles derived from the mitochondrial inner membrane. Figure 1 shows the rate of inactivation of $F_1$ ATPase activity at several

Table I: NBD-Cl Inactivation of ATPase in the Presence of Various Nucleotides[a]

| nucleotide | concn (mM) | $k$ (min$^{-1}$) | $t_{1/2}$ (min) |
|---|---|---|---|
| ATP (EDTA) | 0.05 | 0.120 | 5.8 |
| ATP (EDTA) | 0.25 | 0.112 | 6.2 |
| ADP (EDTA) | 5.0 | 0.111 | 6.2 |
| ATP (EDTA) | 1.0 | 0.0834 | 8.3 |
| ATP (EDTA) | 5.0 | 0.0730 | 9.5 |
| ADP-Mg | 5.0 | 0.0564 | 12.3 |
| $\alpha,\beta$-($CH_2$)-ADP-Mg | 5.0 | 0.0186 | 37.3 |
| $\beta,\gamma$-($CH_2$)-ATP-Mg | 5.0 | 0.0165 | 42.1 |

[a] All experiments employed 100 $\mu$M NBD-Cl. The EDTA concentration was 4 mM if present in the reaction mixture. Magnesium chloride was used to preform nucleotide-magnesium complexes where specifically indicated. Other conditions were 0.2 M sucrose, 50 mM triethanolamine hydrochloride, pH 7.5, at 25 °C, and 2 $\mu$M $F_1$. Values in the first row are from measurements without added nucleotides. The 0.05 mM ATP listed in this row is due to ATP in the $F_1$ preparation which could not be removed without inactivation.

different concentrations of NBD-Cl. The reaction with NBD-Cl was performed under pseudo-first-order conditions and semilogarithmic plots showing the decline in activity as a function of time were characteristically linear over at least 80–90% of the reaction. The reaction order with respect to NBD-Cl was given by the slope of a linear plot of log ($1/t_{1/2}$) against log (NBD-Cl) as previously described (Levy et al., 1963; Scrutton & Utter, 1965). When the data of Figure 1 are plotted in this form, a very good straight line with slope equal to 1.03 was obtained. In agreement with Ferguson et al. (1975), we have found that DTT removes the NBD label and completely reverses the inhibition. For the three different batches of $F_1$ tested in the present work, 1 mM DTT restored full ATPase activity to NBD-labeled $F_1$ within 20 s after addition of the thiol.

The tyrosine residue modified by NBD-Cl is presumably at or near a nucleotide binding site. Added nucleotides would then be expected to afford some protection against inactivation. When inactivation was performed in the presence of different nucleotides and nucleotide concentrations, the extent of protection varied considerably (Table I). In these experiments $F_1$ was equilibrated with nucleotides at concentrations as low as 0.05 mM by gel filtration on Sephadex G-25. A 100-fold increase in ATP concentration caused only a 60% decrease in rate of inactivation, perhaps because the presence of EDTA was required to prevent hydrolysis. However, magnesium complexes of the methylene analogues of either ADP or ATP were capable of slowing inactivation by a factor of approximately 7.

Inactivation of $F_1$ ATPase by NBD-Cl at pH 7.5 is paralleled by formation of a new chromophore with maximal absorbance at about 390 nm and molar extinction coefficient 11 600 (Ferguson et al., 1975). At 100 $\mu$M NBD-Cl, the concentration customarily employed in the present work, ATPase inactivation is 90% complete in about 35 min. Figure 2 shows the incorporation of NBD label into $F_1$ as a function of time, as monitored by the absorbance increase at 390 nm. It is seen that, after 35 min of reaction, 1.0 mol of label has been incorporated. However, observation of the absorbance at still longer times reveals a continuing reaction, approaching incorporation of about 2 mol of label per mol of $F_1$. The semilog plot of the percent of sites unreacted as a function of time, based on two possible reaction sites per $F_1$ molecule, indicates that the time course of reaction is clearly biphasic. Analysis of the rapid and slow kinetic components in terms of a sum of two exponential decay processes yields the second-order rate constants 550 and 59 $M^{-1}$ min$^{-1}$. Incorporation of tritium-labeled NBD-Cl by spinach chloroplast $CF_1$ also exhibits biphasic kinetics and total incorporation of up to at least 1.6 mol of label per mol of $CF_1$ (Cantley & Hammes, 1975). Although reaction of only a single NBD-Cl per $F_1$ is necessary for inactivation of ATPase activity, in agreement with Ferguson et al. (1975), we conclude that reaction of a second NBD-Cl per $F_1$ can and does occur.

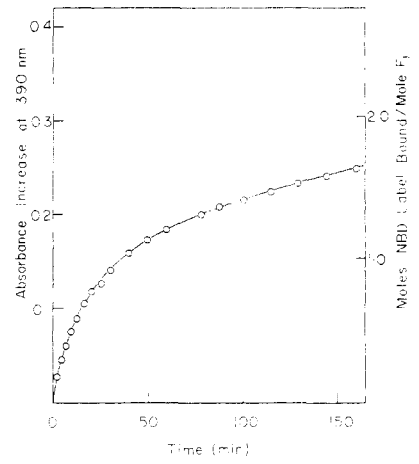


FIGURE 2: Incorporation of NBD label into $F_1$. The reaction mixture contained 4.7 mg of protein in 1.0 mL of buffer with 50 mM triethanolamine hydrochloride, 0.2 M sucrose, 4 mM ATP, 4 mM EDTA, and 100 $\mu$M NBD-Cl, pH 7.5, at 25 °C. Incorporation was monitored by the absorbance increase at 390 nm.

Table II: Effect of NBD-Cl on 2,3-Butanedione Labeling

| sample | % ATPase act. | $k$ for butanedione inact.[a] (min$^{-1}$) | $t_{1/2}$ (min) |
|---|---|---|---|
| $F_1$ | 100 | 0.0459 ± 0.0024 | 15.1 ± 0.8 |
| NBD-$F_1$ | 2.1 | 0.0525 ± 0.0012 | 13.2 ± 0.3 |

[a] ATPase assays were performed in the presence of 2 mM DTT. The concentration of 2,3-butanedione was 4.3 mM and the reaction was performed in buffer containing 50 mM triethanolamine hydrochloride, 0.2 M sucrose, 4 mM ATP, 4 mM EDTA, and 0.1 M borate, pH 8.0, at 25 °C.

Both NBD-Cl and 2,3-butanedione inactivate ATPase activity via covalent modification of $F_1$, presumably at a specific nucleotide binding site or sites. It was therefore of interest to determine if modification by one reagent affects the subsequent reaction of the other. This experiment employed $F_1$ and NBD-$F_1$ prepared as described in Experimental Procedures. The reaction of these samples with 2,3-butanedione at pH 8.0 was then studied. Aliquots of the reaction mixtures were removed after various incubation times and were assayed for ATPase activity in the presence of 2 mM DTT. Thus, the extent of inactivation observed is due only to the extent of reaction with 2,3-butanedione. As shown in Table II, 2,3-butanedione reacts with $F_1$ and NBD-$F_1$ at very similar rates. There is no direct competition between the two labels. Also, any conformational change induced by the NBD label is not sufficient to affect accessibility of 2,3-butanedione to the arginine residue required for ATP hydrolysis.

*Reconstituted ASU Particles.* ASU particles (Racker & Horstman, 1967) are devoid of coupling factor $F_1$ and thus are well suited for reconstitution studies involving chemically modified $F_1$. However, since the final step in preparing ASU particles involves washing with cold 2 M urea, there is some concern of damaging membrane components and perhaps introducing a different rate-limiting step into the overall oxidative phosphorylation process. ASU particles were analyzed for cytochrome content by the spectroscopic technique

Table III: Cytochrome Content of Submitochondrial Particles

| | ASU particles (nmol/mg) | ETPH (nmol/mg) |
|---|---|---|
| Cyt-($a + a_3$) | 2.06 ± 0.05 (1.0)[a] | 1.63 ± 0.04 (1.0)[a] |
| Cyt-*b* | 1.56 ± 0.15 (0.76) | 1.27 ± 0.11 (0.78) |
| Cyt-$c_1$ | 0.47 ± 0.01 (0.23) | 0.36 ± 0.03 (0.22) |
| Cyt-*c* | 0.49 ± 0.03 (0.24) | 0.28 ± 0.04 (0.17) |

[a] Values in parentheses represent the amount of individual cytochromes relative to the amount of cytochrome ($a + a_3$) present.

of Williams (1964). Similar analyses were also performed on ETPH particles. The results presented in Table III show that ASU and ETPH particles have a very similar pattern of cytochrome distribution. The absolute values for different cytochromes, expressed as nmol of cytochrome/mg of particle protein, are uniformly higher for ASU particles than for ETPH since ASU particles are devoid of $F_1$, which normally comprises approximately 20% of the protein of the mitochondrial inner membrane. Respiration rates with succinate as substrate were also directly compared for ETPH and ASU particles reconstituted with OSCP and $F_1$ (see Experimental Procedures). At 37 °C and pH 7.5, respiration rates for $F_1$·ASU and ETPH were, respectively, 326 and 228 ng-atoms of $O_2$/(min·nmol) of cytochrome ($a + a_3$). The smaller value for ETPH reflects the greater retention of respiratory control in these particles than in $F_1$·ASU particles. In the presence of $3 \times 10^{-4}$ M 2,4-dinitrophenol, uncoupler stimulated respiration rates increased to 380 and 402 for $F_1$·ASU and ETPH, respectively. Thus, respiration related processes in $F_1$·ASU particles appear undamaged and certainly are no more rate-limiting than might be the case with ETPH particles.

A direct comparison of respiration rates for $F_1$·ASU and NBD-$F_1$·ASU particles was made using samples in triethanolamine buffer, pH 7.5, at 30 °C with succinate as the substrate (see Experimental Procedures). Basal rates for $F_1$·ASU and NBD-$F_1$·ASU particles were 457 ± 16 and 464 ± 12 ng-atoms of $O_2$/(min·mg), respectively. Respiration of $F_1$·ASU particles in the presence of ADP (5 mM), 2,4-dinitrophenol ($3 \times 10^{-4}$ M), and FCCP ($5 \times 10^{-7}$ M) was stimulated by 1.02-, 1.10-, and 1.09-fold, respectively. Similar measurements on NBD-$F_1$·ASU particles gave stimulation ratios of 0.97, 1.06, and 1.12, respectively. These results emphasize the absence of respiratory control in both reconstituted samples. Furthermore, maximum stimulation of respiration by uncouplers does not exceed approximately 10%. Thus, in assays of ATP synthesis discussed subsequently, respiration is always essentially maximal and invariant.

Concerning the properties of the ASU particles themselves, it should be noted that they have a small residual ATPase activity of about 0.3 μmol/(min·mg), which is less than 5% of the ATPase activity of washed $F_1$·ASU particles. Prior to reconstitution, ASU particles do not catalyze reverse electron transport to a measurable extent (i.e., the activity is ≤0.5 nmol/(min·mg)). Also, ASU particles have a very small apparent activity of about 5 nmol/(min·mg) for net ATP synthesis, which could largely be due to counting error in determining background. Addition of OSCP to ASU particles does not stimulate activity for either reverse electron transport or net ATP synthesis. By these criteria we judge ASU particles to be essentially devoid of $F_1$.

*Nonequivalent Inhibition of ATP Synthesis and Other Reactions Catalyzed by NBD-$F_1$·ASU Particles.* Use of the chemical modification procedures and reconstitution procedures described in Experimental Procedures allows the preparation of submitochondrial particles specifically modified in the $F_1$ moiety and freed of excess modifying agent. Such particles are amenable to assay of ATP synthesis driven by substrate oxidation, ATP-driven reverse electron transport, ATP ⇌ $P_i$ exchange, etc. When NBD-$F_1$ is used to reconstitute ASU particles, the striking result is that net ATP synthesis is very much less inhibited than all other processes assayed.

Table IV: Activities of ASU Particles Reconstituted with $F_1$ or NBD-$F_1$[a]

| measurement | additons to normal medium | act. (nmol/(min·mg of ASU)) $F_1$·ASU | act. (nmol/(min·mg of ASU)) NBD-$F_1$·ASU |
|---|---|---|---|
| reverse electron transport | none | 96.2 | 2.2 |
| | DTT | 95.5 | 78.2 |
| | DTT[b] | 95.1 | 96.1 |
| | oligomycin | ≤0.5 | ≤0.5 |
| ATP ⇌ $P_i$ exchange[c] | none | 116 | 7.5 |
| | DTT | 120 | 77.9 |
| | oligomycin | 6.0 | 7.9 |
| phosphorylation of ADP | none | 163 | 110 |
| | DTT | 179 | 187 |
| | oligomycin | 12.3 | 8.7 |
| phosphorylation of IDP[d] | none | 63.8 | 41.7 |
| | DTT | 56.8 | 68.3 |
| | oligomycin | 3.5 | 5.3 |
| phosphorylation of ADP (minus ATP trapping system)[e] | none | 139 | 79.9 |
| | rotenone + antimycin | 21.4 | 20.4 |

[a] ATPase activities of soluble coupling factors were: $F_1$, 51.0 μmol/(min·mg); NBD-$F_1$, 1.1 μmol/(min·mg). All assays were at 30 °C and pH 7.5 except the reverse electron transport assays, which were at 30 °C and pH 7.8. The concentration of DTT, when present, was 1 mM. [b] A separate control with DTT added to soluble coupling factors prior to reconstitution of ASU particles. [c] Measurements of this exchange were performed in the presence of 4 μM rotenone + 4 μM antimycin. [d] For these experiments, gel filtration was used to equilibrate soluble coupling factors with buffer containing 4 mM ITP instead of ATP. Reconstitution of ASU particles was then performed in the presence of buffer containing 2 mM ITP. [e] Hexokinase and glucose were omitted from the assay medium and nucleotides were separated by chromatography on PEI paper.

Results of a typical set of experiments are summarized in Table IV. Preparation of NBD-$F_1$ as described in Experimental Procedures gives virtually complete inactivation of ATPase activity, with less than 3% of the initial activity remaining. The same level of inhibition carries over to measurements of ATP-driven reverse electron transport by NBD-$F_1$·ASU particles. In the presence of oligomycin (2 μg/mg of protein), the reverse electron transport activity is essentially zero for both $F_1$·ASU and NBD-$F_1$·ASU particles. ATP ⇌ $P_i$ exchange by NBD-$F_1$·ASU particles is also inhibited to the level of oligomycin sensitivity. The control $F_1$·ASU particles catalyze reverse electron transport and ATP ⇌ $P_i$ exchange (and net ATP synthesis) with activities similar to those obtainable with ETPH particles. However, for the experiments of Table IV, net ATP synthesis by NBD-$F_1$·ASU particles is about two-thirds of that found for $F_1$·ASU particles. ATP synthesis for both samples is 90–95% oligomycin sensitive. Exactly the same pattern is obtained when the substrate for phosphorylation is IDP instead of ADP.

One advantage of the NBD label is its ready removability by DTT. The presence of 1 mM DTT completely regenerates ATPase activity in NBD-$F_1$, providing assurance that the native protein structure is generally undamaged by the

Table V: Effect of Malonate or Antimycin on NBD-Cl-Induced Inhibition of ATP Synthesis[a]

| sample | net ATP synth (nmol/(min·mg of ASU)) | ratio of NBD-treated/control × 100 |
|---|---|---|
| $F_1$·ASU | 170.2 | 40.7 |
| NBD-$F_1$·ASU | 69.2 | |
| $F_1$·ASU + malonate | 57.4 | 37.1 |
| NBD-$F_1$·ASU + malonate | 21.3 | |
| $F_1$·ASU + antimycin | 35.7 | 48.7 |
| NBD-$F_1$·ASU + antimycin | 17.4 | |

[a] Measurements were performed at 30 °C and pH 7.5 in the presence of a hexokinase-glucose trap, as described in Experimental Procedures. The concentration of malonate during the assay was 25 mM, and that of antimycin was 1.25 μM.

modification reaction. Furthermore, DTT treatment of NBD-$F_1$ prior to reconstitution with ASU particles (Table IV) produces complete regeneration of reverse electron transport activity. Addition of DTT to NBD-$F_1$·ASU particles during assay of reverse electron transport gives regeneration of activity within 20 s after addition of the thiol, the activity then remaining constant for at least 4 min. But the activity thus regenerated is reproducibly 80–85% of the control. This may reflect some change in accessibility of a labeled site to DTT after coupling factor has been rebound to the membrane. A similar effect is noted in regenerating ATP $\rightleftharpoons$ $P_i$ exchange activity by NBD-$F_1$·ASU particles. However, the partial inhibition of net ATP synthesis with NBD-$F_1$·ASU particles is completely reversed by DTT treatment of the reconstituted particles.

The partial inhibition of ATP synthesis by NBD-Cl (when other activities are fully inhibited) has been confirmed in studies on three different batches of $F_1$. Excess coupling factor can be removed from reconstituted particles by ultracentrifugation and resuspension in fresh buffer. Such washing does not alter the pattern of results shown in Table IV, nor does freezing (−70 °C) and thawing of reconstituted particles. Furthermore, the use of a hexokinase–glucose trap for ATP is not necessary for the demonstration of net ATP synthesis by reconstituted particles. Nucleotide separations on PEI paper (see Experimental Procedures) allow direct evaluation of incorporation of radioactive inorganic phosphate into ATP. Data obtained in the absence of the ATP trapping system (Table IV) also show that ATP synthesis by NBD-$F_1$·ASU particles is only partially inhibited. However, under such conditions, a slightly increased amount of rotenone–antimycin insensitive ATP formation is also observed.

Reducing the overall rate of electron transfer by the presence of malonate or antimycin has little effect on the relative activity of NBD-$F_1$·ASU particles in ATP synthesis (Table V). The activity of NBD-$F_1$·ASU compared with $F_1$·ASU particles maintains a relatively constant ratio, even when ATP synthesis in both samples is extensively inhibited by either malonate or antimycin. The partial inhibition of ATP synthesis by NBD-$F_1$·ASU particles appears to be correlated with the partial modification of a second tyrosine residue per $F_1$ molecule. The NBD-$F_1$ samples employed in the present work have normally contained from about 1.35 to 1.6 mol of NBD label per mol of $F_1$, as judged from spectral data of the type given in Figure 2.

*ASU Particles Reconstituted with 2,3-Butanedione Treated $F_1$.* Modification of arginine residues in soluble $F_1$ by dione reagents such as 2,3-butanedione causes concomitant inactivation of ATPase activity (Marcus et al., 1976) and ATP $\rightleftharpoons$ $P_i$ exchange activity (Frigeri et al., 1977). Direct information concerning the essential role of these arginine residues in ATP synthesis has been obtained from experiments analogous to those previously described involving NBD-Cl. Figure 3 shows the results of an experiment designed to probe possible differential effects of butanedione inhibition. Coupling factor $F_1$ was modified to varying degrees by 2,3-butanedione by varying the total incubation time with the dione. Reactions catalyzed by membrane-bound $F_1$ employed ASU particles reconstituted with appropriate 2,3-butanedione modified samples of $F_1$. It is seen that the time course for inactivation of ATPase activity of soluble $F_1$ closely parallels that for inactivation of ATP synthesis by reconstituted ASU particles. However, there is a much faster loss of activity for ATP driven reverse electron transport. For these experiments, the longest exposure of $F_1$ to 2,3-butanedione was 105 min. It should be emphasized that, for ASU particles reconstituted with the $F_1$ treated 105 min, the residual activity for ATP synthesis was still more than 90% sensitive to inhibition by oligomycin (4 μg/mg of protein) or by FCCP ($5 \times 10^{-7}$ M final concentration).

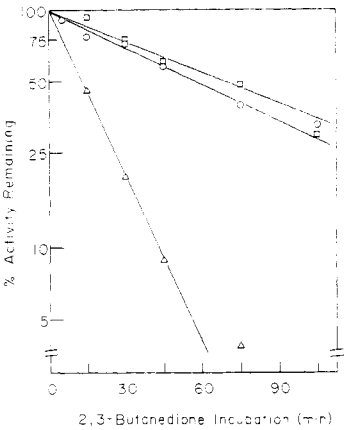


FIGURE 3: Effect of labeling $F_1$ with 2,3-butanedione on various reactions catalyzed. Activities assayed were ATP synthesis (□), ATPase of soluble $F_1$ (○), and ATP-driven reverse electron transport (△). The 100% activity values were: ATP synthesis, 114 nmol/(min·mg); ATPase, 66.1 μmol/(min·mg); reverse electron transport, 81.8 nmol/(min·mg). Purified $F_1$ was incubated with butanedione for the time periods shown and then passed through Sephadex and recombined with ASU particles.

The data in Figure 3 clearly demonstrate the presence of arginine residues essential for net ATP synthesis (top curve) and for reverse electron transport (bottom curve). Furthermore, the rate of loss of these activities differs by fivefold. But ATPase inactivation of isolated $F_1$ occurs at essentially the same rate as loss of activity for net ATP synthesis, unlike the results described for NBD-Cl modification. We cannot exclude the possibility that $F_1$ labeled by butanedione at an ATPase site might participate in a transfer reaction following reconstitution, resulting in migration of label to an ATP $\rightleftharpoons$ $P_i$ exchange site of the type described by Frigeri et al. (1977).

## Discussion

The reaction of NBD-Cl with coupling factor $F_1$ from beef heart mitochondria has been reported to inactivate ATPase activity through specific modification of tyrosine in the β subunit (Ferguson et al., 1975). We have further characterized this labeling and have studied its effects on various reactions normally catalyzed by membrane-bound $F_1$. The results in Figure 1 show a rapid inactivation of $F_1$ ATPase activity on incubation at pH 7.5 with NBD-Cl. This inactivation is first order with respect to NBD-Cl. The additional presence of magnesium complexes of either α,β-($CH_2$) ADP or β,γ-($CH_2$)–ATP provides marked protection against the inacti-

vation, which is consistent with the NBD-Cl reaction occurring at or near a nucleotide binding site. In contrast to the results of Ferguson et al. (1975), we find that the labeling of $F_1$ by NBD-Cl is not limited to 1 mol of label per mol of $F_1$. Rather, the incorporation is biphasic and approaches 2 mol of label per mol of $F_1$. This is quite reasonable in view of other recent labeling studies on $F_1$. For example, the photoaffinity label 8-azido-ATP reacts specifically with the $\beta$ subunits of beef heart $F_1$ (Wagenvoord et al., 1977) and the chemically reactive ATP analogue Nbs$^6$ITP, 6-[(3-carboxy-4-nitrophenyl)-thio]-9-$\beta$-D-ribofuranosylpurine 5′-triphosphate, specifically labels the $\beta$ subunits of $F_1$ from a bacterial plasma membrane (Hulla et al., 1978). In both cases the stoichiometry of reaction is 2 mol of label per mol of $F_1$. Furthermore, there are 2 aurovertin binding sites per $F_1$, aurovertin binding is biphasic (Chang & Penefsky, 1974; van de Stadt & van Dam, 1974), and each $\beta$ subunit contains 1 aurovertin binding site (Verschoor et al., 1977). Considering these facts and the presence of a twofold molecular symmetry axis (Amzel & Pedersen, 1978), it seems likely that the 2 tyrosine residues modified by NBD-Cl are on separate $\beta$ subunits within the native structure.

The reconstituted ASU particles used in the present studies have proven to be very satisfactory for the assay of net ATP synthesis, reverse electron transport, and ATP $\rightleftarrows$ $P_i$ exchange. In these assays, $F_1$·ASU particles yielded specific activities comparable to those reported by other workers for beef heart ETPH particles prepared according to Beyer (1967). Specifically, for ETPH at 30 °C and pH 7.4 to 7.6 with succinate as the substrate, recent literature shows maximal activities for reverse electron transport of 80–120 nmol/(min·mg) (Rottenberg & Gutman, 1977) and 80.1 ± 5.9 nmol/(min·mg) (Leimgruber & Senior, 1976b) and maximal activities for ATP synthesis of 152–173 nmol/(min·mg) (Hackney & Boyer, 1978) and 168 nmol/(min·mg) (Penefsky, 1974). Under similar assay conditions, our values for $F_1$·ASU particles (Table V) are 96.2 nmol/(min·mg) for reverse electron transport and 163 nmol/(min·mg) net ATP synthesis. Thus, turnover during both ATP utilization and ATP synthesis appears normal in the reconstituted particles.

Moudrianakis and co-workers as well as Boyer and co-workers have recently proposed an alternating site model for oxidative phosphorylation (Adolfsen & Moudrianakis, 1976; Kayalar et al., 1977). This model proposes the participation of two identical enzyme catalytic sites, such that during oxidative phosphorylation the binding of ADP and $P_i$ at one site is necessary for subsequent events leading to release of ATP from the second site. It regards the energy derived from electron transfer reactions as driving a conformational change to an enzyme state capable of forming ATP from ADP and $P_i$ at the catalytic site without additional energy input. Also, ATP hydrolysis is viewed as a reversal of the events for ATP synthesis.

It is difficult to rationalize our present results in terms of such a model. If there is compulsory interaction between two sites in the alternating site model, then blocking one site stops the cycle. If the blocking is achieved by covalent modification, then one site is "tagged" throughout a series of measurements. An agent like NBD-Cl which blocks ATPase activity should also block ATP synthesis, if these reactions occur by simple dynamic reversal.

Findings in several laboratories indicate that coupling factors $F_1$ and $CF_1$ undergo conformational transitions related to their function (Chang & Penefsky, 1974; Ryrie & Jagendorf, 1972; McCarty & Fagan, 1973). During recent years, the role of negative cooperativity and half-of-the-sites reactivity in enzyme regulation has received considerable attention (Levitzki & Koshland, 1976). The action of coupling factor $F_1$ during oxidative phosphorylation may well involve negative interactions between catalytic subunits, as suggested by Boyer and co-workers (cf. Hackney & Boyer, 1978) and by Cross & Kohlbrenner (1978). Our results with reconstituted ASU particles would be consistent with a model in which there are two catalytic sites per $F_1$ molecule, only one of which is involved in net ATP synthesis at any given time. This would constitute a half-of-the-sites reactivity pattern if the sites were intrinsically identical but subject to interconversion induced by nucleotide binding pattern or through interaction with other protein factors such as the ATPase inhibitor, OSCP, or specific membrane sector proteins in $F_0$. We cannot rule out possible explanations based on negative cooperativity. However, our findings are directly explained if there are intrinsically different catalytic sites or if sites involved in ATP synthesis and ATP utilization are partially overlapping.

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# Proton and Phosphorus Nuclear Magnetic Resonance Studies of Ribonuclease $T_1$†

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ABSTRACT: $^1H$ and $^{31}P$ nuclear magnetic resonance (NMR) studies of ribonuclease (RNase) $T_1$ are reported. Assignments of the C2-H proton resonances of the three histidine residues were made using a tritium labeling technique which is a combination of differential tritium exchange at the C2-H position of histidine [Matsuo, H., Ohe, M., Sakiyama, F., & Narita, K. (1972) *J. Biochem. (Tokyo) 72*, 1057; Ohe, M., Matsuo, H., Sakiyama, F., & Narita, K. (1974) *J. Biochem. (Tokyo) 75*, 1197] and $^1H$ NMR of a differentially deuterated protein. $^1H$ NMR data taken in the absence and presence of guanosine 3′-monophosphate (3′-GMP), a strong competitive inhibitor to the enzyme, were used along with $^{31}P$ NMR spectra of the inhibitor to provide information on the structure of the active site of the enzyme. It was concluded that histidine-40 along with a carboxyl group which is probably that of glutamic acid-58 is responsible for the catalytic action of the enzyme. The structure of the active site of RNase $T_1$ is in a marked contrast with that of RNase A where two histidine residues are known to act as a general acid and general base to conduct the catalytic action. Interaction involving histidine-92 and N-7 of 3′-GMP through a hydrogen bond is most likely responsible for the enzyme–inhibitor binding. A scheme of the active site and of the interaction of the enzyme with 3′-GMP is presented on the basis of the present experimental results.

Ribonuclease $T_1$ (RNase $T_1$;[1] EC 2.7.7.26) is highly specific to the 3′-phosphodiester bridge of a guanosine base in the RNA chain (Uchida & Egami, 1971; Takahashi, 1974). In RNase $T_1$, which is composed of 104 amino acid residues with the two disulfide bridges, the three histidine RNase occur at positions 27, 40, and 92 (Takahashi, 1971). Two of them, His-40 and His-92, along with one glutamic acid (Glu-58) and one arginine (Arg-77), have been suggested to be in or near the active site, participating in either binding or catalytic action of the enzyme (Takahashi et al., 1967; Takahashi, 1970, 1973).

It has been well established that $^1H$ NMR peaks of histidines give invaluable information about the structure of proteins in solution once each individual resonance can be assigned to a particular histidine residue in the amino acid sequence (Roberts & Jardetzky, 1970; Markley, 1975a). A first NMR study of RNase $T_1$ was reported by Ruterjans and

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[1] Abbreviations used: RNase, ribonuclease; GMP, guanosine monophosphate; CMP, cytosine monophosphate; NMR, nuclear magnetic resonance; FT, Fourier transform; $Me_4Si$, tetramethylsilane; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

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