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Reaction of membrane-bound F_1-adenosine triphosphatase of *Escherichia coli* with chemical ligands and the asymmetry of β subunits

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The three β subunits of the isolated *Escherichia coli* F_1-ATPase react independently with chemical reagents (Stan-Lotter, H. and Bragg, P.D. (1986) Arch. Biochem. Biophys. 284, 116–120). Thus, one β subunit is readily cross-linked to the ε subunit, Another reacts with *N,N'*-dicyclohexylcarbodiimide (DCCD), and the third one is modified on a lysine residue by 4-chloro-7-nitrobenzofurazan (NbfCl). The binding site for the ATP analog, 2-azido-ATP, was not associated with a specific type of β subunit (Bragg, P.D. and Hou, C. (1989) Biochim. Biophys. Acta 974, 24–29). We now show that this binding site is a catalytic site as opposed to a noncatalytic nucleotide-binding site. NbfCl reacted with a tyrosine residue on the DCCD-reacting β subunit in contrast to the different subunit location of the lysine residue labeled by the reagent. Thus, O to N transfer of the Nbf group in the free F_1-ATPase involves transfer between subunits. The chemical labelling pattern of membrane-bound F_1-ATPase differed from that of free F_1. The strict asymmetry of labeling of the free F_1-ATPase was not observed. Thus, double labeling of β subunits by several reagents was found. This suggests that the asymmetry was not induced by chemical modification, but is inherent in the structure of the ATPase.

Introduction

The F_1-ATPase of *E. coli* consists of five different types of subunit, designated α–ε, in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. This enzyme is attached to the F_0 portion in the membrane. F_0 is composed of three further types of subunit [1]. The F_1F_0 complex can carry out ATP hydrolysis and synthesis. It seems likely that these processes involve two or three catalytic sites acting sequentially [1,2]. Three non-catalytic adenine nucleotide binding sites are present in the enzyme [1].

The β subunits of the free F_1-ATPase show an interesting type of asymmetry, as first recognized by Lötscher and Capaldi [3] and extended by Stan-Lotter and Bragg [4–6]. Thus, one-third of the β subunits react with NbfCl and IAANS but not with DCCD. Another one-third of the β subunits react with DCCD but not with NbfCl or IAANS. The remaining one-third of the β subunits does not react with the above reagents but is readily cross-linked to the ε subunit by EDC. It is not known whether this behavior is a consequence of a permanent structural asymmetry in the F_1-ATPase as possibly suggested by X-ray crystallography [7], an asymmetry induced by chemical labeling, or a measure of a transient asymmetry occurring during the catalytic cycle. Furthermore, it is not known if the asymmetry observed with the free F_1-ATPase is present in the membrane-bound enzyme.

In the present paper we have clarified some labeling properties of the free F_1-ATPase and have also examined the possible asymmetry of the β subunits in the membrane-bound enzyme. In contrast to the situation where the Nbf group was attached to a lysine residue investigated in previous studies, the tyrosine-bound Nbf group was found to be present on the DCCD-reactive β subunit, thereby suggesting that intersubunit transfer of the Nbf group occurs. The strict asymmetry of the β subunits of free F_1 does not appear to be maintained in the membrane-bound enzyme. Therefore, it is unlikely

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAANS, 2-(4-iodoacetamidoaniline)naphthalene-6-sulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; NbfCl, 4-chloro-7-nitrobenzofurazan.

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that the modifying reagents are inducing the observed asymmetry of the free F_1-ATPase, but that it is a feature of the structure of the enzyme. We previously showed with free F_1 that the photoactivatable analog of ATP, 2-azido-ATP, did not label one type of β subunit specifically [8]. This fact, together with the demonstration in the present paper that 2-azido-ATP labels catalytic, as opposed to non-catalytic, adenine nucleotide-binding sites, suggests that the catalytic site does not have a specific relationship to a particular type of β subunit, but that it cycles between different β subunits as suggested by Gresser et al. [2].

Materials and Methods

Preparation of F_1-ATPase and urea-stripped membranes

The F_1-ATPase and urea-stripped membranes were prepared from *E. coli* CM2786. This strain carries the plasmid pBJC706 which contains an insert of the entire *unc* operon encoding the subunits of the ATP synthase [9]. Cell membranes from this strain have amplified levels of the synthase. The F_1-ATPase was prepared from washed cell membranes as described previously [10]. Cell membranes from which the F_1-ATPase had been removed by washing with low ionic strength buffer were further stripped of peripheral membrane proteins by treatment with 4 M urea in a buffer of 1 mM Tris-HCl/10% (v/v) glycerol/0.5 mM EDTA/0.1 mM dithiothreitol (pH 7.5) for 30 min at 20 °C. The stripped membraneas were sedimented by centrifuging at 250000 $\times g$ for 1 h. They were washed twice by sedimentation from the same buffer without urea, and were suspended for storage at −70 °C in 50 mM Hepes-KOH/10% (v/v) glycerol/10 mM $MgCl_2$ (pH 7.5) at a protein concentration of 26 mg/ml.

Chemical modification of free and membrane-bound F_1-ATPase

Free F_1-ATPase (6–10 mg protein/ml) was modified with 1 mM NbfCl or 10 mM IAANS for 1 h at 30 °C in a buffer containing 50 mM triethanolamine/0.5 mM EDTA/10% (v/v) glycerol (pH 7.5). Excess reagent was removed on centrifuged 1 ml columns of Sephadex G-50 [11] equilibrated with the same buffer, except when the Nbf group was to be transferred from tyrosine to lysine residues. Then the pH of the column buffer was 9 and the enzyme was incubated subsequently at 37 °C for 1 h. For DCCD treatment, the enzyme was incubated for 1 h at 20 °C with 0.5 mM DCCD in 100 mM Mops/10% (v/v) glycerol/0.5 mM EDTA (pH 6.5).

Modified or non-modified F_1-ATPase was rebound to urea-stripped membranes as follows. The F_1-ATPase (2.5–5 mg protein/ml) was incubated with urea-stripped membranes (2.5–5 mg protein/ml) in 50 mM Hepes-KOH/10 mM $MgCl_2$ (pH 7.5) for 30 min at 20 °C. The reconstituted membranes were sedimented by centrifugation at 220000 $\times g$ for 1 h, and washed by resedimentation from Hepes/$MgCl_2$ buffer. The reconstituted membranes or washed native membranes were suspended in the appropriate buffer at a protein concentration of 2.2 – 5 mg/ml for further treatment. The chemical modification by DCCD, NbfCl and IAANS was carried out using the same buffer as with the free F_1-ATPase, but with the reagents at a concentration of 1 mM, 10 mM and 20 mM, respectively. Transfer of the Nbf group from tyrosine to lysine residues was carried out as above. Following treatment, the membrane suspension was diluted 100-fold with buffer and the membranes sedimented by ultracentrifugation prior to examination by gel electrophoresis.

The F_1-ATPase was photolabeled with 2-azido-ATP as follows. The enzyme (6.9 mg protein/ml) was incubated in the dark with 0.2 mM 2-azido-ATP for 30 min at 20 °C in a buffer of 50 mM triethanolamine/10% (v/v) glycerol/0.1 mM EDTA (pH 7.5). Excess nucleotide was removed on a 1 ml centrifuged column of Sephadex G-50 equilibrated with the same buffer. The enzyme was then irradiated at 20 °C for 15 min at a distance of 15 cm from a Spectroline EF 280C ultraviolet light source. In some experiments the enzyme was incubated with 1 mM ATP/0.7 mM $MgCl_2$ at 20 °C for 1 h, and the excess reagents removed, prior to incubating with 2-azido-ATP. In other experiments, the 2-azido-ATP-treated enzyme was incubated for 30 min at 20 °C in the dark with ATP/$MgCl_2$ prior to irradiation.

For treatment of washed or reconstituted membranes, 200 μM 2-azido-ATP was added and incubated for 10 min prior to irradiation with ultraviolet light for 5 min. This treatment was repeated for a total of eight to ten times. The treated membranes were resedimented by ultracentrifugation.

Crosslinking of β to ε subunits with EDC in the free F_1-ATPase was carried out as described in Ref. 8.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [12]. Isoelectric focusing gels were run in one dimension using the O'Farrell system [13], modified as described in Ref. 14. Proteins were stained with Coomassie blue [15]. Gels containing radioisotopes were exposed at −70 °C to Kodak XAR-5 film.

Assays

ATPase activity was measured at 37 °C with Malachite Green. Protein was determined by the method of Bradford [17] with bovine serum albumin as a standard.

Chemicals

The following materials were supplied by the companies indicated. Sigma: DCCD, NbfCl, EDC, ATP. Pharmacia: ampholytes, Sephadex G-50. Amersham: [^{14}C] DCCD. Research Products International Corporation: [^{14}C] NbfCl.

The ATP analog, 2-azido-ATP was prepared from 2-azido-AMP, kindly provided by Dr. Paul Boyer, as described by Melese and Boyer [18].

Results

Isoelectric focusing gels have been used extensively in the analysis of our data on the chemical modification of the β subunits. A more extensive discussion of their use and the interpretation of the resulting gels is given in Stan-Lotter and Bragg [4].

Although the labeling of the free F_1-ATPase of *E. coli* has been extensively examined, information on the reactions of chemical reagents with the membrane-bound enzyme is incomplete. Pougeois et al. [19] showed that at pH 6.5, but not at pH 8.0, β subunits of the membrane ATPase were labelled. The fluorescent sulfhydryl-reacting compound IAANS labeled α, γ and ϵ subunits in the membrane-bound F_1. By contrast, there was greater labeling of β subunits in free F_1 [4]. However, there is no information on the sites of interaction of NbfCl and 2-azido-ATP with the membrane enzyme of *E. coli,* although Dalbon et al. [20] have shown that β subunits are labeled by 2-azido-ADP in submitochondrial particles.

Labeling of ATPase with NbfCl

Prior to examination of the labeling of membrane-bound F_1-ATPase by NbfCl, the reaction of this compound with free F_1 was further characterized. In previous studies we had allowed the reagent to react initially with a tyrosine residue of the β subunit and then induced transfer of the Nbf group from this to a lysine residue at pH 9. This was done on the supposition that the *N*-Nbf group would be more stable than the *O*-Nbf label. As shown in Fig. 1, *O*-Nbf labeled F_1 is sufficiently stable for subsequent manipulations to be carried out. In the experiment shown in Fig. 1, free F_1-ATPase was reacted with [^{14}C]NbfCl. A portion of the enzyme was then incubated at pH 9 to induce transfer of the Nbf group from a tyrosine to a lysine residue. As expected, in contrast to the *O*-Nbf group, the *N*-Nbf group was not removed by dithiothreitol or 2-mercaptoethanol. Both the O- and N-labeled enzymes were subsequently treated with DCCD and the pattern of labeling of β subunits examined on isoelectric focusing gels (Fig. 1). As shown in lane 1, the untreated β subunit moved as a major single species together with a minor satellite band which migrated as if it possessed an extra negative charge. The cause of the satellite species is not known, but deamidation of the subunit seems a plausible explanation. The appearance of the β subunit on Coomassie blue stained gels following formation of the *O*-Nbf derivative was unchanged (lane 2) because substitution of the tyrosine residue did not alter the charge on the subunit. The incorporation of the [^{14}C]Nbf group by both the major and the satellite bands is seen in lane 2 of the radioautograph. Lane 4 shows an increase in the intensity of the band in the -1 position when the O to N transfer of the Nbf group had been brought about. This transfer resulted in the loss of one positive charge by the subunit. The transfer is shown clearly by the movement of radiolabel in the radioautograph. This also demonstrates that O to N transfer was incomplete. These and previous results have shown that only one of the three β subunits reacts with NbfCl. As we have shown previously [5], subsequent treatment of *N*-Nbf labeled F_1 with DCCD results in the reaction of a β subunit with DCCD, which is different from that carrying the *N*-Nbf group. This is shown in Fig. 1, lane 5. In the Coomassie blue stained gel, a new band appears at the $+1$ position due to the loss of one negative charge as a carboxyl group on one of the β subunits was substituted by DCCD. A lesser amount of the disubstituted subunit is seen in the $+2$ position. The radioautograph shows no movement of the major labeled band of the *N*-Nbf labeled subunit, but some of the *O*-Nbf labeled band has lost a negative charge and now migrates in the $+1$ position. Thus, as previously found, the DCCD reactive β subunit is different from the *N*-Nbf labeled subunit. The reaction of the *O*-Nbf labeled β subunit with DCCD is seen clearly in Fig. 1, lane 3, where a substantial portion of the *O*-Nbf-labeled subunit now migrates in the $+1$ position.

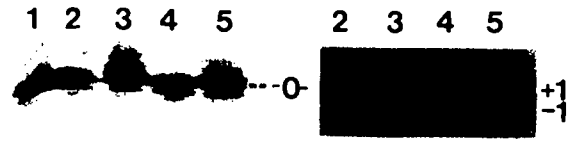

Fig. 1. Labeling of *O*- or *N*-Nbf modified F_1-ATPase by DCCD. The experimental procedures are described in Materials and Methods. The ATPase was modified with [^{14}C]NbfCl to give the *O*-Nbf derivative. A portion of the enzyme was treated at pH 9 to transfer the Nbf group to a lysine amino group. The two types of modified enzyme were subsequently treated with DCCD. The labeled β subunits were separated on an isoelectric focusing gel and stained with Coomassie blue (left-hand panel), or exposed to an X-ray film (right-hand panel). The number of positive or negative charges due to incorporation of labels is indicated. The lanes are: 1, untreated F_1; 2, *O*-[^{14}C]Nbf-treated F_1; 3, *O*-[^{14}C]Nbf-treated F_1 subsequently treated with DCCD; 4, *N*-[^{14}C]Nbf-treated F_1; 5, *N*-[^{14}C]NBf-treated F_1 subsequently treated with DCCD.

The labeling behaviour of β subunits of membrane-bound F_1 with NbfCl (and DCCD) was now examined. For these and subsequent experiments two types of membrane preparation were used. Strain CM2786 contains the plasmid pBJC706. This multicopy plasmid

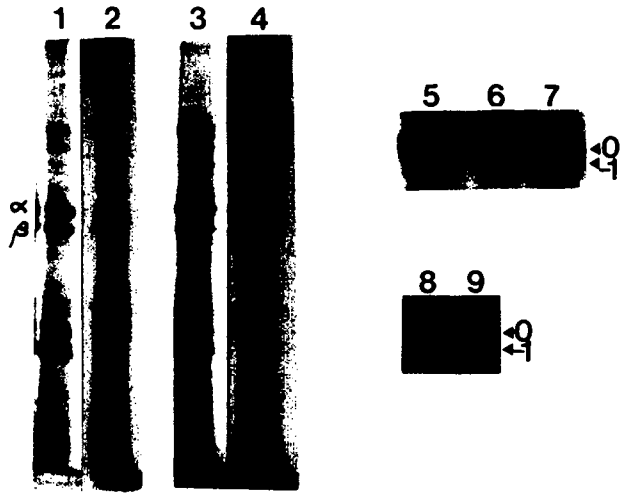

Fig. 2. Labeling of membrane-bound F_1-ATPase with NbfCl and DCCD. The experimental procedures are described in Materials and Methods. Lanes 1, 2, SDS polyacrylamide gel electrophoresis of *O*-[^{14}C]Nbf-labeled membranes; 3, 4, *N*-[^{14}C]Nbf-labeled washed membranes. Lanes 1 and 3 were stained with Coomassie blue. Lanes 2 and 4 are autoradiographs. Lanes 5–9 are isoelectric focusing gels of β subunits from treated enzymes stained with Coomassie blue (5–7) or autoradiographs (8, 9). Lane 5, untreated F_1; 6, F_1 treated with 0.5 mM DCCD; 7, F_1 treated with 1 mM NbfCl and the Nbf group subsequently transferred to a lysine residue; 8, *O*-[^{14}C]Nbf-labeled F_1; 9, *N*-[^{14}C]Nbf-labeled F_1. The number of positive or negative charges due to incorporation of label is indicated.

encodes the entire ATP synthase complex and results in membranes of CM2786 possessing high levels of subunits of the complex [9]. The α and β subunits of the F_1-ATPase are readily visible on SDS-polyacrylamide gels. Washed membranes of CM2786 were treated directly with chemical labels in one set of experiments. For other experiments the F_1-ATPase from this strain was purified to homogeneity, treated with the chemical label, and then rebound to F_1-ATPase-stripped membranes of CM2786. The reloaded membranes were subsequently treated with another label. We were not able to detect any difference in the behavior between the two types of preparation in any of the experiments described in this paper.

Treatment of washed membranes with NbfCl and DCCD showed that the β subunits of F_1-ATPase were preferentially labeled (Fig. 2, lanes 1–4). Other membrane proteins were labeled by the reagents, but this did not interfere with the interpretation of the experiments. The effect of modification of the β subunits by reaction with DCCD and NbfCl on their position of migration on isoelectric focusing gels is shown in Fig. 2; lanes 5–7. As with free F_1, reaction with DCCD results in the removal of one negative charge. Reaction with NbfCl and subsequent treatment at pH 9 yields a β subunit with one less positive charge. One β subunit of the available three subunits is modified by each reagent. That O to N transfer of the [^{14}C]Nbf group occurs in membrane-bound F_1 is shown in the radioautographs (Fig. 2, lanes 8, 9). The O-labeling does not change the charge of the β subunits (0 position, Fig. 2, lane 8). After transfer to a lysine residue at alkaline pH, with loss of a positive charge, the labeled subunit migrates in the -1 position.

Labeling of ATPase with 2-azido-ATP

In previous work we showed that there was a single high-affinity site for 2-azido-ATP on the isolated F_1-ATPase of *E. coli* [8]. This site was on β subunits. However, we did not show if this site was catalytic or noncatalytic. The procedure of Wise et al. [21] was used to clarify this point. The F_1-ATPase was incubated with 2-azido-ATP, and then with ATP in the presence of $MgCl_2$, before photolytic labeling. Incubation with ATP/$MgCl_2$ displaces 2-azido-ATP from catalytic sites so that noncatalytic sites only are labeled on photolysis. Catalytic sites were labeled by first loading catalytic and noncatalytic sites with ATP/$MgCl_2$ and subsequently displacing the nucleotide from catalytic sites with 2-azido-ATP prior to photolysis. The results of these experiments are shown in Fig. 3. Treatment of F_1-ATPase resulted in the labeling of the β subunit only. As shown by isoelectric focusing (lane 2) the main new band was that formed by the incorporation of the diphosphate (-2 position). Some triphosphate was present, as also were bands in the -4 and -5 positions which could be due to the labeling by a second molecule of nucleotide. The latter bands and that of the diphosphate were reduced under conditions where the catalytic site was loaded with ATP prior to photolysis (lane 3). Therefore, under the conditions of our experiments, 2-azido-ATP was predominantly bound at a catalytic site. This is consistent with the observed hydrolysis of this compound to the diphosphate.

Labeling of membrane-bound F_1-ATPase by 2-azido-ATP was inefficient. As shown in Fig. 3, lane 7, the diphosphate was the major product of incorporation into the β subunit. Incorporation was obtained only by

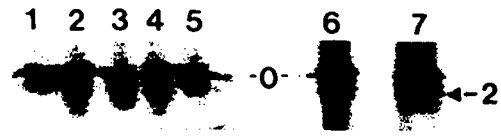

Fig. 3. Reaction of 2-azido-ATP with free and membrane-bound F_1-ATPase. The experimental procedures are described in Materials and Methods. The enzyme was treated with 2-azido-ATP and the β subunits separated by isoelectric focusing. The gels were stained with Coomassie blue. The number of positive or negative charges due to incorporation of label is indicated. Lane 1, untreated free F_1; 2, F_1 treated with 2-azido-ATP and irradiated; 3, F_1 treated with 2-azido-ATP, and then with ATP/$MgCl_2$ before irradiation; 4, F_1 treated with ATP/$MgCl_2$, then with 2-azido-ATP before irradiation; 5, F_1 treated with 2-azido-ATP but not irradiated. Lane 6, untreated membrane-bound F_1; 7, membrane-bound F_1 treated ten times with 2-azido-ATP, and irradiated following each addition.

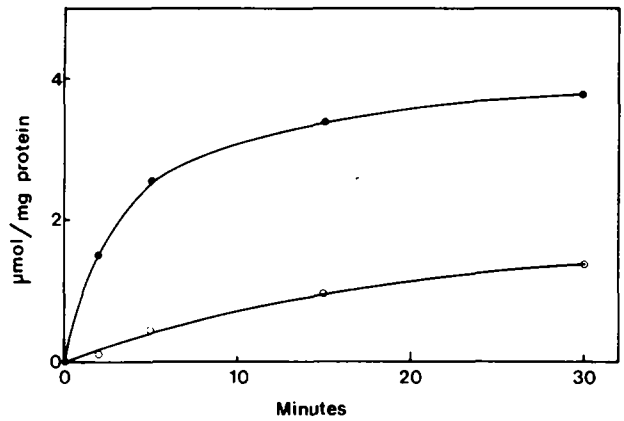

Fig. 4. Hydrolysis of ATP and 2-azido-ATP by membrane-bound F_1-ATPase. ATP or 2-azido-ATP were incubated at a concentration of 0.2 mM with 0.1 mM $MgCl_2$ in 50 mM triethanolamine-HCl/10% glycerol (pH 7.5). Washed membranes were added and samples removed at intervals for the analysis of liberated phosphate by the Malachite Green method. Liberated phosphate (μ mol/mg membrane protein) is plotted as a function of time of incubation. ●, ATP; ○, 2-azido-ATP.

repeated exposure of the membrane to fresh aliquots of the nucleotide. In the experiment shown, ten additions were made. Significant labeling was observed with three additions, however. The difficulty of labeling was surprising since 2-azido-ATP was hydrolyzed by membrane-bound F_1-ATPase at a significant rate (Fig. 4). The inefficiency of labeling was not due to ultraviolet light absorption by the membranes since addition of membranes did not effect the photolytic labeling of free F_1-ATPase by 2-azido-ATP.

Formation of membrane-bound βε crosslinked F_1-ATPase

Lötscher et al. [22], first showed that treatment of free F_1-ATPase with the water-soluble carbodiimide EDC resulted in crosslinking of β and ε subunits. They [3] and we [4–6] subsequently showed that the crosslinkable β subunit had a unique reactivity with chemical probes. Neither group reported on the possibility that a $\beta\varepsilon$ crosslink could be formed with membrane-bound F_1-ATPase. A $\beta\varepsilon$ crosslink can be formed on treatment of membrane-bound F_1-ATPase with EDC (data not shown). However, a more convenient way is to treat F_1 with EDC to form the crosslink (Fig. 5, lanes 1, 2) and then to rebind the treated F_1-ATPase to F_1-stripped membranes (lanes 3, 4). The advantage of this method lies in the fact that the removal of F_1 from the membranes with 4 M urea, also removes other periperal proteins thereby making the SDS-polyacrylamide gel patterns easier to interpret (lanes 3, 4).

Double labeling of β subunits of F_1-ATPase

In the free F_1-ATPase, the β subunit forming a crosslink with the ε subunit is uniquely characterized by an inability to form the lysyl derivative with NbfCl and by a lack of reaction with DCCD [3,4–6]. Membrane-bound F_1-ATPase crosslinked with EDC was treated with [^{14}C]DCCD or [^{14}C]NbfCl. Part of the sample treated with the latter reagent was incubated at pH 9 to induce migration of the Nbf group from a tyrosine to a lysine residue. The presence of radioactivity in the $\beta\varepsilon$ crosslinked subunits was examined by radioautography (Fig. 5, lanes 5–7). The β subunit band was heavily labeled by both DCCD and NbfCl. However, the DCCD did not label the $\beta\varepsilon$ product significantly, although NbfCl reacted with it, particularly as the *O*-derivative.

As shown above, DCCD will label the same β subunit in free F_1-ATPase as that which is labeled by NbfCl on a tyrosine residue (Fig. 1). That this is true for the membrane-bound F_1-ATPase is demonstrated in Fig. 6A. Urea-stripped membranes were reloaded with DCCD-labeled F_1 (lane 2) or [^{14}C]*O*-Nbf-labeled F_1 (lane 4), and subsequently treated with [^{14}C]NbfCl or nonradioactive DCCD, respectively (lanes 3, 5). The radioautograph of lane 4 showed that [^{14}C]NbfCl predominantly labeled the tyrosine residue (O position), although some *N*-derivative (-1 position) was present also. Subsequent labeling by DCCD (lane 5) resulted in a portion of the labeling now being found in the $+1$ position, thereby indicating that *O*-Nbf labeled F_1 had become labeled by DCCD also (reaction of the latter reagent reduces the negative charge on the β subunit by one charge). Treatment of the DCCD-labeled enzyme on the membranes with NbfCl (lane 3), showed that the radioactivity of the *O*-Nbf group had become incorporated into the DCCD-labeled β subunit in the $+1$ position.

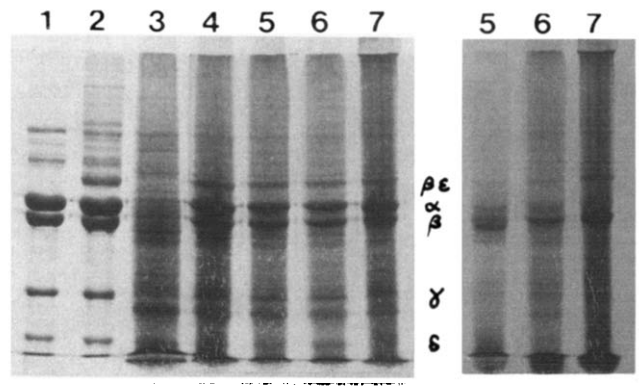

Fig. 5. Reaction of EDC-crosslinked membrane-bound F_1-ATPase with [^{14}C]DCCD and [^{14}C]NbfCl. The experimental procedures are described in Materials and Methods. Free F_1 (lane 1) was treated with EDC to generate the $\beta\varepsilon$ crosslink (lane 2). The treated F_1 was incubated with urea-stripped membranes (lanes 3) and the rebound F_1 (lane 4) subsequently treated with [^{14}C]DCCD (lane 5) or [^{14}C]NbfCl. The *O*-Nbf label (lane 7) was transferred at pH 9 to give the *N*-Nbf substituent (lane 6). The subunits of the treated enzyme were resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (left-hand panel) or exposed to an X-ray film (right-hand panel).

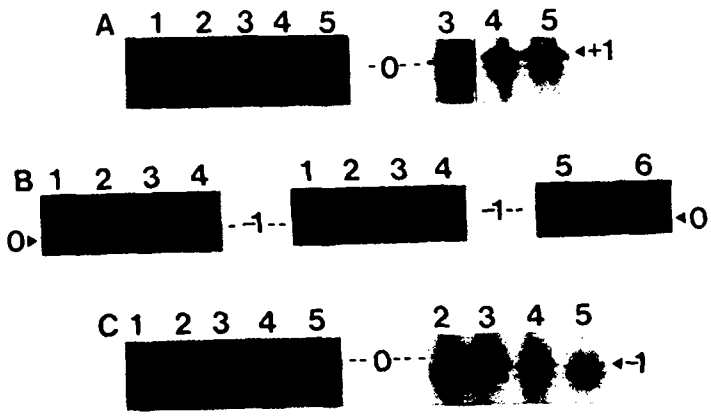

Fig. 6. Double-labeling of β subunits of membrane-bound F_1-ATPase with DCCD, NbfCl, IAANS and 2-azido-ATP. The experimental procedures are described in Materials and Methods. The β subunits were resolved on isoelectric focusing gels and stained with Coomassie blue or exposed to X-ray film. The number of positive or negative charges due to incorporation of label is indicated. Panel A; lane 1, urea-stripped membranes reloaded with untreated F_1; 2, urea-stripped membranes reloaded with DCCD-labeled F_1; 3, as lane 2 but subsequently treated to give the *O*-[^{14}C]Nbf-labeled enzyme; 4, urea-stripped membranes reloaded with *O*-[^{14}C]Nbf-labeled F_1; 5, as lane 4 but subsequently treated with DCCD. Left-hand side, Coomassie blue stained; right-hand side, autoradiography. Panel B: lane 1, urea-stripped membrane reloaded with [^{14}C]DCCD-labeled F_1; 2, as lane 1 but subsequently treated to give the *N*-Nbf labeled enzyme; 3, as lane 1 but subsequently treated with 2-azido-ATP and irradiated; 4, as lane 1 but subsequently treated with IAANS. Lane 5, as lane 1; lane 6, as lane 4. Left-hand panel, Coomassie blue stained; center and right-hand panels, radioautograph. Panel C: lane 1, urea-stripped membranes reloaded with untreated F_1; 2, urea-stripped membranes reloaded with N-[^{14}C]Nbf-labeled F_1; 3, as lane 2 but subsequently treated with DCCD; 4, as lane 2 but subsequently treated with IAANS; 5, as lane 2 but subsequently treated with 2-azido-ATP and irradiated. Left-hand panel, Coomassie blue stained; right-hand panel, autoradiograph.

The reactivity of the [^{14}C]DCCD-labeled β subunit of membrane-bound F_1-ATPase with 2-azido-ATP, IAANS, and with NbfCl (labeling a lysine residue) is shown in Fig. 6B. Treatment of DCCD-labeled enzyme with NbfCl, and transference of the Nbf group to a lysine residue, showed that a portion of the DCCD-labeled β subunit migrated from the +1 to the zero position on the elimination of a single positive charge as Nbf reacted with the lysine residue (Fig. 6B, lane 2). Reaction of the DCCD-labeled β with the negatively charged IAANS and with 2-azido-ATP resulted in the acquisition of one and two negative charges by this subunit, respectively (Fig. 6B, lanes 4, 3). The radioautographs of Fig. 6B, lanes 5, 6 confirm the modification of the [^{14}C]DCCD-labeled β subunit by IAANS.

In Fig. 6C the ability of DCCD, IAANS and 2-azido-ATP to modify the N-[^{14}C]Nbf-labeled β subunit is investigated. There is little evidence of further modification of this subunit, except in the case of 2-azido-ATP where radioactive bands in the −2 and −3 positions are seen (Fig. 6C, lane 5). These are likely the adenosine mono and diphosphates labeling the *N*-Nbf-labeled β subunit. The formation of the monophosphate from 2-azido-ATP in labeling experiments was observed previously with chloroplast F_1 by Melese et al. [23].

Discussion

As outlined in the Introduction, the three β subunits of free F_1-ATPase can be categorized into (a) DCCD-reacting, (b) IAANS and *N*-Nbf reacting, and (c) crosslinkable to the ε subunit by EDC. A β subunit reacting in one of these classes (a)–(c) is not modifiable by reagents affecting the other two classes. The rigid separation of the reactivity of the β subunits to the different reagents was found not to hold in the case of the membrane-bound F_1-ATPase. Thus, the β subunits reacting with DCCD could be subsequently modified by NbfCl, IAANS and 2-azido-ATP. The *O*- and *N*-Nbf-modified β subunits could be labeled by DCCD and 2-azido-ATP, respectively. The β subunit which can be crosslinked to the ε subunit could be substituted by NbfCl. The further labeling of *O*-Nbf labeled β subunit by DCCD is not surprising since, as described in this paper, we have observed this with the free F_1-ATPase. Furthermore, as we have shown previously [8], 2-azido-ATP does not discriminate between the different β subunits. However, the loss of discrimination in the membrane-bound F_1-ATPase between *N*-Nbf- and DCCD-labeled β subunits, and between *N*-Nbf- and ε-reacting β subunits is significant. This result suggests that the 'type' of the β subunit is not generated by reaction with the reagent per se, but already exists in the arrangement of the β subunits. These are clearly in a different conformation in the membrane-bound F_1 compared with the free ATPase. Although it is also possible that in the course of the catalytic cycle the same β subunit successively acquires the properties of each of the types, thus permitting double-labeling, we have not, in contrast to the results of Melese and Boyer [18] with free chloroplast F_1, been able to turn over the membrane-bound enzyme with ATP to confirm this possibility.

A further interesting finding is the fact that the Nbf group attached to a tyrosine residue will label the DCCD-reactive β subunit in free F_1, whereas the Nbf group attached to a lysine residue will not do so. It is unlikely that the lack of labeling of the DCCD-substituted β by the *N*-derivative is due to steric reasons. The polypeptide chain folding for the β subunit predicted by Duncan et al. [24] places the Nbf-binding residues distant from the DCCD-reactive residues. This leads to the intriguing possibility that transfer of the Nbf group from tyrosine to lysine residues is a transfer between subunits and not within the same polypeptide chain.

Finally, we have extended our previous data on the reaction of the free F_1-ATPase with 2-azido-ATP. Our initial studies showed that 1 mol 2-azido-ATP bound per mol F_1 [8]. In the present paper we show that the site labeled is a catalytic site on a β subunit. In the previous work we found that 2-azido-ATP reacted indiscriminately with all three 'types' of β subunits. This is probably true of the membrane-bound ATPase, although we have no information on the ε-crosslinkable β subunit. These results are consistent with the model of Boyer and co-workers [2], in which each β subunit becomes the active site in turn duringthe catalytic cycle.

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