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INACTIVATION OF *RHODOSPIRILLUM RUBRUM* COUPLING FACTOR BY 7-CHLORO-4-NITROBENZO-2-OXA-1,3-DIAZOLE

MODIFICATION OF A TYROSINE PROTECTED BY PHOSPHATE

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Chemical modification of *Rhodospirillum rubrum* chromatophores by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) results in inactivation of photophosphorylation, Mg^{2+}-ATPase, oxidative phosphorylation and ATP-driven transhydrogenase, with apparent first-order kinetics. Other energy-linked reactions such as light-driven transhydrogenase and light-dependent proton uptake were insensitive to NBD-Cl. The Ca^{2+}-ATPase activity of the soluble coupling factor from chromatophores (*R. rubrum* F_1) was inactivated by NBD-Cl with kinetics resembling those described for Mg^{2+}-ATPase and photophosphorylation activities of chromatophores. Both NBD-chromatophores and NBD-*R. rubrum* F_1 fully recovered their activities when subjected to thiolysis by dithioerythritol. Phosphoryl transfer reactions of chromatophores and Ca^{2+}-ATPase activity of *R. rubrum* F_1 were fully protected by 5 mM P_i against modification by NBD-Cl. ADP or ATP afforded partial protection. Analysis of the protection of Ca^{2+}-ATPase activity by P_i indicated that NBD-Cl and P_i are mutually exclusive ligands. Spectroscopic studies revealed that tyrosine and sulfhydryl residues in *R. rubrum* F_1 underwent modification by NBD-Cl. However, the inactivation was only related to the modification of tyrosine groups.

Introduction

The bacterium *Rhodospirillum rubrum* can grow aerobically in the dark or anaerobically in the light. Cells grown in the light contain an oxidoreductive chain that is coupled to the synthesis of ATP in the dark [1]. Different lines of evidence suggest that the coupling factor catalyzing oxidative and photosynthetic phosphorylation in chromatophores is the same protein [2,3].

The coupling factor from *R. rubrum* chromatophores (*R. rubrum* F_1) is a large molecule (M_r = 350000) [4] composed of five different subunits and is functionally linked to a membrane sector (*R. rubrum* F_0) composing the ATP synthetase complex. Upon detachment from the membrane *R. rubrum* F_1 shows a Ca^{2+}-dependent ATPase activity which is sensitive to aurovertin [5,6] and efrapeptin [3] but insensitive to oligomycin and DCCD at low concentrations (high concentrations of DCCD block a functional carboxyl group in the β-subunit of *R. rubrum* F_1 [7]). The whole complex (*R. rubrum* F_0-*R. rubrum* F_1) has been resolved from the membrane, purified and actively reconstituted in artificial liposomes [8–10].

By chemical modification with group-specific reagents, it was possible to identify several amino acid residues essential for the activity of coupling

Abbreviations: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; BChl, bacteriochlorophyll.

factors of oxidative and photosynthetic phosphorylation [11,12]. Some models, showing the arrangement of amino acid residues at the active site, have been proposed [11,12]. Using arginine-specific reagents, we have shown that the sites for ATP synthesis and hydrolysis of ATP in *R. rubrum* F_1 are partially different regarding the arginine residue modified by butanedione or phenylglyoxal [13]. Two distinct carboxyl residues essential for *R. rubrum* F_1 activity have been recently identified by chemical modification with DCCD and Woodward's reagent K [7].

NBD-Cl has been extensively used for the modification of tyrosine, sulfhydryl and lysine residues in a number of enzymes. The particular group reacting with NBD-Cl within the protein molecule can be identified on the basis of the optical properties of the NBD derivatives [14–16]. This reagent modifies, at neutral or slightly alkaline pH values, essential tyrosine residues in coupling factors from mitochondria [15], *Escherichia coli* [17] and chloroplasts [18] and presumably in the $(Na^+ + K^+)$-ATPase from electroplax [19]. Reports from different laboratories suggest that in the case of mitochondrial [15,20–22] and chloroplast [23] coupling factors, the tyrosine residues reacting with NBD-Cl are not at the active site. However, in the case of $(Na^+ + K^+)$-ATPase the group reacting with NBD-Cl was related to the active site [19,24]. In *E. coli* coupling factor [17] the experimental evidence is consistent with the location of the NBD-Cl-modified residues at the active site or very close to it. Modification of *R. rubrum* chromatophores [25] and *R. rubrum* F_1 [26] by NBD-Cl has also been reported.

This paper is concerned with the effects of ligands such as P_i, ADP and ATP on the modification of *R. rubrum* chromatophores and *R. rubrum* F_1 by NBD-Cl. A characterization of the residues modified by NBD-Cl in *R. rubrum* F_1 is presented.

Materials and Methods

Materials

R. rubrum cells (Van Niel Strain S.) were grown anaerobically in the light as described earlier [27]. Harvesting of the cells and preparation of chromatophores were as described previously [5]. *R. rubrum* F_1 was extracted from chromatophores (37 mg BChl) at room temperature as described earlier [28] except that the suspension medium (200 ml) contained 20 mM Tris-H_2SO_4 (pH 7.8), 10% glycerol, 1 mM EDTA and 1 mM ATP (buffer A). The following purification steps were carried out at 0°C. The supernatant was centrifuged at 40000 $\times g$ for 45 min and loaded on a DEAE-cellulose column (25 × 2.5 cm). The column was washed with 200 ml of buffer A and *R. rubrum* F_1 was eluted with 150 ml of buffer A containing 200 mM ammonium sulfate. Fractions with Ca^{2+}-ATPase were pooled and fractionated between 30 and 60% saturation of ammonium sulfate. The precipitate resuspended in a small volume (3–4 ml) of buffer A was applied to a CL-6B Sepharose column (80 × 2 cm) which was eluted with the same buffer. Fractions with ATPase activity were pooled, precipitated at 60% saturation of ammonium sulfate and stored at 0–4°C. Usually, 20 mg of *R. rubrum* F_1 with a specific activity of 11–15 μmol ATP/min per mg protein were obtained.

Fresh solutions of NBD-Cl were prepared in ethanol and protected from the light. All other chemicals were of analytical grade.

Methods

Treatment of chromatophores with NBD-Cl was carried out in the dark at 25°C in a medium (1 ml) containing 50 mM NaOH-Tricine (pH 8), 250 mM sucrose, 5 mM $MgCl_2$ and chromatophores (200 μg BChl). Samples were withdrawn at different incubation times and diluted about 20-fold in the appropriate assay medium. Mg^{2+}-ATPase, phenazine methosulfate-induced photophosphorylation, transhydrogenase driven by light or by ATP and light-induced proton uptake were assayed as described previously [3] except that Tris buffer was replaced by Tricine at the same pH and concentration. Oxidative phosphorylation was measured in the dark at 25°C as described earlier [5]. BChl was determined using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ at 772 nm [29].

Chemical modification of *R. rubrum* F_1 (200 μg/ml) with NBD-Cl was accomplished as described for chromatophores. Preparation of iodoacetamide-*R. rubrum* F_1 was carried out at 25°C in a medium (250 μl) containing 40 mM NaOH-Tricine (pH 8), 10 mM iodoacetamide and 600 μg *R. rubrum* F_1. Excess iodoacetamide was

eliminated by the column centrifugation-filtration method [30]. Ca^{2+}-ATPase activity was measured as reported earlier [3]. Spectroscopic properties of the complex between NBD-Cl and *R. rubrum* F_1 or iodoacetamide-*R. rubrum* F_1 were monitored on an Aminco DW-2a spectrophotometer fitted with a MIDAN 'T' microprocessor and circulating water for temperature control.

Protein was determined according to the method of Lowry et al. [31] using bovine serum albumin as standard.

Results

Modification of R. rubrum chromatophores and solubilized coupling factor with NBD-Cl

Uncoupling, electron-transport blockage or phosphoryl-transfer inhibition are possible mechanisms by which ATP synthesis can be prevented in *R. rubrum* chromatophores. An evaluation of the effects of a given inhibitor on photoreactions, ATP-driven reactions and phosphoryl-transfer reactions gives information for discerning the mechanism by which ATP synthesis is impaired [3]. The energy-linked transhydrogenase activity in *R. rubrum* membranes can be driven by light or by hydrolysis of ATP. Fig. 1 shows that NBD-Cl treatment of chromatophores inactivated the ATP-dependent transhydrogenase but had no effect on the light-driven reaction. Chromatophores derived from photosynthetically grown cells catalyze oxidative phosphorylation in the dark [1]. Fig. 1 shows that oxidative phosphorylation was similarly inactivated by treatment with NBD-Cl.

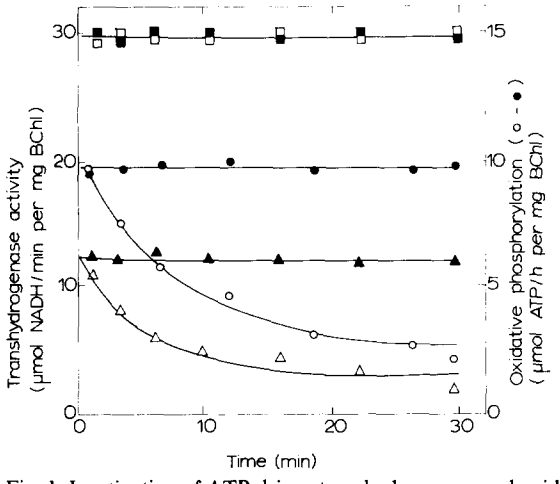

Fig. 1. Inactivation of ATP-driven transhydrogenase and oxidative phosphorylation by NBD-Cl in *R. rubrum* chromatophores. Modification of chromatophores with 50 μM NBD-Cl and measurement of light-dependent transhydrogenase (□——□), ATP-driven transhydrogenase (△——△) and oxidative phosphorylation (○——○) were carried out as described in the text. Closed symbols correspond to control activities measured in chromatophores that were incubated in the absence of NBD-Cl.

Inactivations of the ATP-dependent transhydrogenase and oxidative phosphorylation were fully reversed by subsequent treatment of chromatophores with 500 μM dithioerythritol.

Light-dependent proton uptake by chromatophores is sensitive to uncouplers and electron-transport inhibitors [32]. Treatment of chromatophores with NBD-Cl did not alter the light-induced pH changes measured afterwards (not shown).

ATP synthesis and ATP hydrolysis catalyzed by *R. rubrum* chromatophores are inactivated by 100 μM NBD-Cl according to a single-exponential decay [25]. We studied the effect of several concentrations of NBD-Cl (between 5 and 50 μM) and found that the inactivation of the reactions mentioned above was first order with respect to NBD-Cl (data not shown). Under our experimental conditions, the inactivation of chromatophores by NBD-Cl levels off at prolonged incubation times. Pertinent controls suggested that the levelling off was due to depletion of NBD-Cl from the incubation mixture, presumably by reaction with nucleophilic groups exposed on the membrane surface.

Johansson et al. [4] have purified *R. rubrum* F_1 from an acetone powder extract obtained from *R. rubrum* chromatophores. The purified *R. rubrum* F_1 is endowed with a Ca^{2+}-dependent ATPase activity and reconstitutes Mg^{2+}-dependent ATPase and photophosphorylation activities when added back to *R. rubrum* F_1-depleted chromatophores [4]. Alternatively, *R. rubrum* F_1 can be extracted with chloroform from an aqueous suspension of chromatophores [28]. The *R. rubrum* F_1 partition into the aqueous phase from which it can be further purified [28]. When NBD-chromatophores were extracted with chloroform an inactive Ca^{2+}-ATPase was recovered. *R. rubrum* F_1 remained inactive after ammonium sulfate fractionation and

TABLE I

CHLOROFORM EXTRACTION OF COUPLING FACTOR FROM NBD-CHROMATOPHORES

Treatment of chromatophores with 100 μM NBD-Cl for 60 min, and assay of Mg^{2+}-ATPase and Ca^{2+}-ATPase were performed as described in Materials and Methods. Chloroform extraction of *R. rubrum* F_1 from NBD-chromatophores (NBD-Chr) and control chromatophores (Control-Chr) was carried out as described in the text except that the whole procedure was scaled down to one-tenth.

Step	ATPase activity (μmol ATP/min per mg protein)	
	Control-Chr	NBD-Chr
Chromatophore Mg^{2+}-ATPase activity	3	0.1
Ca^{2+}-ATPase in 200000 × *g* supernatant after $CHCl_3$ extraction	5.8	0.03
Ca^{2+}-ATPase in 60% ammonium sulfate fraction	9.6	0.07
Ca^{2+}-ATPase after 15 min of addition of 500 μM dithioerythritol	9.7	5
Ca^{2+}-ATPase activity after 30 min of addition of 500 μM dithioerythritol	9.5	9.6

was reactivated upon addition of dithioerythritol. These results are summarized in Table I.

It was previously reported that NBD-Cl inactivates Ca^{2+}-ATPase in *R. rubrum* F_1 [26]. We confirmed that observation and found that inactivation by 10, 20, 30 and 40 μM NBD-Cl followed a single-exponential decay with an order of reaction for NBD-Cl close to unity.

Effect of the ligands P_i, ADP and ATP during modification of chromatophores and R. rubrum F_1 by NBD-Cl

When modification of chromatophores by NBD-Cl was carried out in the presence of 20 mM P_i photophosphorylation was totally protected (Fig. 2). ADP or ATP (20 mM) afforded partial protection. Similar results were observed with the Mg^{2+}-ATPase activity of chromatophores. Even with P_i concentrations as low as 1 mM considerable protection of both activities was obtained (data not shown). The Ca^{2+}-ATPase activity of the purified coupling factor was also totally protected

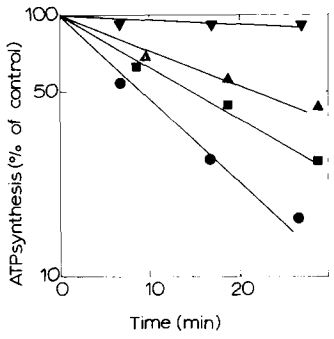

Fig. 2. Effect of ADP, ATP and P_i on the modification of *R. rubrum* chromatophores by NBD-Cl. Chromatophores were incubated with 50 μM NBD-Cl and the following additions: none (●———●), 20 mM ADP (■———■), 20 mM ATP (▲———▲) and 20 mM P_i (▼———▼). NBD-Cl treatment and photophosphorylation assay were carried out as described in the text. Activity of the control was 560 μmol ATP/per mg BChl.

by 5 mM P_i and partially protected by 20 mM ADP or ATP (data not shown). With lower concentrations of P_i the pattern of partial protection shown in Fig. 3 was obtained. Plotting the data according to the method of Scrutton and Utter [33] gave a straight line intersecting the ordinate at zero and a K_d for P_i of 0.7 mM (Fig. 3, inset).

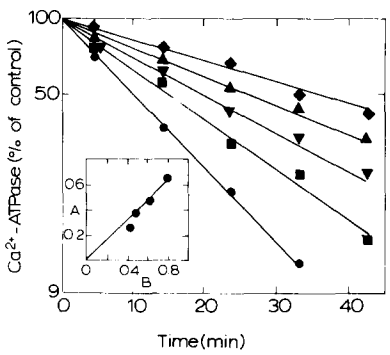

Fig. 3. Effect of P_i on the modification of *R. rubrum* F_1 by NBD-Cl. *R. rubrum* F_1 was incubated with 20 μM NBD-Cl and the following P_i concentrations: none (●———●), 0.4 (■———■), 0.8 (▼———▼), 1.2 (▲———▲) and 1.6 mM (◆———◆). At the indicated times, Ca^{2+}-ATPase activity was measured in aliquots containing 2 μg protein as described in the text. Inset, '*A*': K'_{P_i}/K'_0, and '*B*': $(1 - K'_{P_i}/K'_0)/[P_i]$ where K'_{P_i} and K'_0 are the pseudo-first-order constants for the inactivation of *R. rubrum* F_1 in the absence and presence of P_i, respectively.

Residues modified by NBD-Cl on R. rubrum F_1

NBD-Cl reacts with tyrosine, cysteine and lysine residues in proteins and model compounds. The adducts formed have characteristic absorbance maxima at 385 nm (NBD-Tyr), 420 nm (NBD-Cys) and 475 nm (NBD-Lys) [15].

The inactivation of *R. rubrum* F_1 by NBD-Cl was associated with the appearance of a broad absorbance peak between 380 and 440 nm (Fig. 4A). This result indicates that, under the experimental conditions described in Fig. 4A, NBD-Cl did not modify lysine residues in *R. rubrum* F_1 and shows that both tyrosine and cysteine residues underwent nitroaryl ether modification.

R. rubrum F_1 is a sulfhydryl enzyme. Incubation of purified *R. rubrum* F_1 with 10 mM *N*-ethylmaleimide resulted in 75% inactivation of its Ca^{2+}-ATPase activity (Fig. 5).

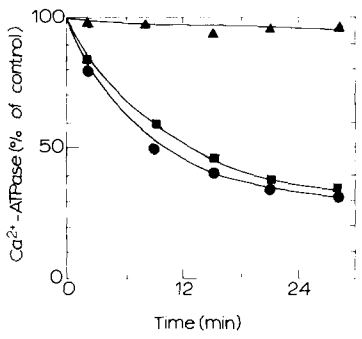

Fig. 5. Modification of *R. rubrum* F_1 with *N*-ethylmaleimide and iodoacetamide. *R. rubrum* F_1 (200 μg) was incubated with 10 mM *N*-ethylmaleimide (■———■), 10 mM iodoacetamide (▲———▲) or 10 mM *N*-ethylmaleimide plus 2 mM P_i (●———●) at 25°C in 40 mM NaOH-Tricine (pH 8). Ca^{2+}-ATPase was assayed in aliquots (2 μg protein) at the stated times. Activity of the control was 12 μmol ATP/min per mg protein.

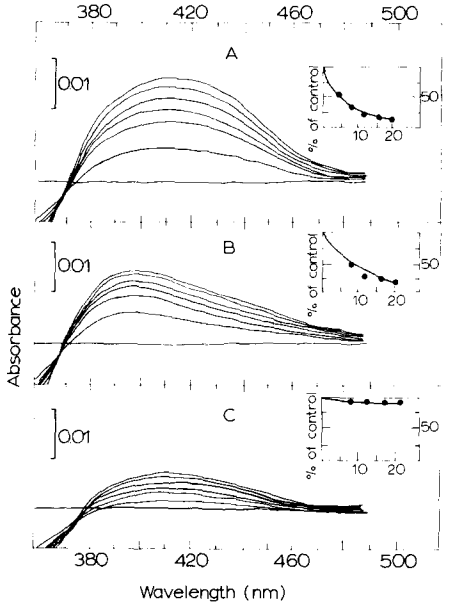

Fig. 4. Spectroscopic analysis of the reaction of *R. rubrum* F_1 and iodoacetamide-*R. rubrum* F_1 with NBD-Cl. (A) *R. rubrum* F_1 (1.5 μM) was incubated with 20 μM NBD-Cl as described in the text and the difference absorbance spectra against a reference containing only 20 μM NBD-Cl were recorded at 2, 6, 10, 14, 18 and 23 min, in aliquots (2 μg protein) taken at the same time Ca^{2+}-ATPase activity was assayed. Ca^{2+}-ATPase activity of the control was 12.5 μmol ATP/min per mg protein. (B) As A except that *R. rubrum* F_1 (1.42 μM) was pretreated with 10 mM iodoacetamide for 45 min as described in the text. (C) As B except that 10 mM P_i was present during treatment with NBD-Cl.

Inactivation by *N*-ethylmaleimide was slow ($t_{1/2}$ = 13 min at 25°C) and was not protected by P_i. Under similar conditions, *R. rubrum* F_1 was insensitive to treatment with iodoacetamide (Fig. 5).

The sensitivity of *R. rubrum* F_1 to *N*-ethylmaleimide and the spectral data presented in Fig. 4A indicated that the inhibition of *R. rubrum* F_1 activity by NBD-Cl could have been caused by the modification of tyrosine or cysteine residues or both. To discern which group was related to the inactivation by NBD-Cl, *R. rubrum* F_1 was treated with iodoacetamide and subsequently exposed to NBD-Cl. The resulting spectrum is shown in Fig. 4B. The blockage of nonessential sulfhydryl groups in *R. rubrum* F_1 by iodoacetamide produced a blue shift (390 nm) in the spectrum of the NBD-Cl-modified enzyme. The appearance of the peak at 390 nm was paralleled by the inactivation of the Ca^{2+}-ATPase (Fig. 4B, inset).

When the modification of iodoacetamide-*R. rubrum* F_1 with NBD-Cl was carried out in the presence of 10 mM P_i the absorbance enhancement at 390 nm was largely prevented (Fig. 4C) and the Ca^{2+}-ATPase activity was fully protected (Fig. 4C, inset). As noted in Fig. 4C, iodoacetamide-*R. rubrum* F_1 contained some free sulfhydryl groups which could react with NBD-Cl but were not essential for the Ca^{2+}-ATPase activity.

Discussion

The inactivation of ATP synthesis, Mg^{2+}-ATPase, oxidative phosphorylation and ATP-driven transhydrogenase in *R. rubrum* chromatophores by NBD-Cl (Ref. 25 and this paper) suggests that the ATPase complex is the primary target of the reagent. Moreover, the lack of inactivation of light-driven transhydrogenase and light-dependent proton uptake demonstrates that neither the electron transport nor the H^+ permeability of the membrane was impaired in NBD-chromatophores.

The results in Table I show that *R. rubrum* F_1 extracted from NBD-chromatophores had an inactive ATPase that could be reactivated by dithioerythritol treatment. This observation reinforces the suggestion that NBD-Cl reacts with an amino acid residue of *R. rubrum* F_1. On the other hand, the possibility that the membrane-bound sector of the complex (*R. rubrum* F_0) may be labeled by NBD-Cl was not ruled out. A tyrosine residue of the DCCD-binding proteolipid was suggested to participate in the rate-limiting step of proton conduction in the thermophilic bacterium PS3 [34].

An interesting observation encountered in the study of ligands as protectors of the inactivation by NBD-Cl was the low degree of protection by ADP and ATP compared with the complete protection by P_i (Fig. 2).

As the strong protection by P_i seemed to be particularly meaningful we studied the variation of the pseudo-first-order constant for the inactivation of *R. rubrum* F_1 as a function of P_i concentrations. A plot according to the method of Scrutton and Utter [33] showed a straight line that passed through the origin, implying that the P_i-enzyme complex cannot be inactivated by NBD-Cl and therefore suggesting that P_i and NBD-Cl are mutually exclusive ligands. From the slope of the straight line an apparent dissociation constant for P_i (K_d^{app}) of 0.7 mM (at pH 8) was calculated. If the charged species of P_i interacting with *R. rubrum* F_1 were the monovalent anion, as in the case of mitochondrial F_1 [35], the true value for K_d^{app} should be 95 μM.

A careful spectral analysis is required to establish the specificity of the reaction of NBD-Cl with a given protein [36]. This approach has been particularly useful in the case of the modification of mitochondrial coupling factor with NBD-Cl [15,37] in which the initial incorporation of the NBD moiety into a tyrosine residue as well as its transfer to a sulfhydryl or lysine residue, under appropriate conditions, were characterized by the spectral and fluorescence properties of the adducts formed.

When *R. rubrum* F_1 reacted with NBD-Cl a broad absorbance peak between 380 and 440 nm was observed (Fig. 4A). It is worth noting that the reaction of mitochondrial F_1 with NBD-Cl, under conditions comparable to those described in Fig. 4A, did not show any absorbance in the region of 420 nm [15], suggesting the lack of NBD-Cl-reactive sulfhydryl residues in this coupling factor.

The difference spectra obtained (Fig. 4A) indicate that *R. rubrum* F_1 contains sulfhydryl and tyrosine residues that react with NBD-Cl. From the chemical modification of *R. rubrum* F_1 with iodoacetamide and *N*-ethylmaleimide (Fig. 5) we concluded that *R. rubrum* F_1 contains both reactive and essential sulfhydryl groups. When the reactive sulfhydryl groups on *R. rubrum* F_1 were blocked by iodoacetamide the maximum in the absorbance spectrum shifted to 390 nm. The appearance of the peak at 390 nm was accompanied by a loss in Ca^{2+}-ATPase activity, suggesting that iodoacetamide-*R. rubrum* F_1 reacts freely with NBD-Cl and that an essential tyrosine residue is blocked.

The fact that P_i, in addition to protecting iodoacetamide-*R. rubrum* F_1 against inactivation by NBD-Cl, also prevented the absorbance enhancement at 390 nm reinforces the conclusion that P_i and NBD-Cl are mutually exclusive ligands at *R. rubrum* F_1.

The residual absorbance of iodoacetamide-*R. rubrum* F_1 modified by NBD-Cl in the presence of P_i (Fig. 4C) suggests that some sulfhydryl residues react with NBD-Cl but are not essential for activity.

The observation that the soluble and membrane-bound activities of *R. rubrum* F_1 presented similar patterns of inactivation by NBD-Cl, reversion by dithioerythritol and protection by ligands, suggests that NBD-Cl modifies the same residue on *R. rubrum* F_1 regardless of whether it is soluble or bound to the membrane.

The role of the NBD-Cl-reactive tyrosine res-

idue in mitochondrial F_1 has been called a mystery in a recent report [38]. The earliest studies on the effects of NBD-Cl on mitochondrial F_1 revealed that the tyrosine residue modified by NBD-Cl was not located at the nucleotide-binding site [15].

Other results with coupling factors from mitochondria [15,20–22] and chloroplasts [23] supported the original observation made by Ferguson et al. [15], suggesting that NBD-Cl inactivates these coupling factors in an indirect manner. Two distinct tyrosine residues on the β-subunit of mitochondrial F_1 were detected by Esch and Allison [21], one at the nucleotide-binding site reacting with *p*-fluorosulfonylbenzoyl-5′-adenosine and another labeled by NBD-Cl and supposedly remote from the catalytic site. Relevant to the possible role of the NBD-Cl-reactive tyrosine residue are some observations encountered in the studies of P_i binding to mitochondrial F_1. The interaction of P_i with mitochondrial F_1 was studied to some extent [30,35] but as yet the nature of the P_i-binding sites remains unknown. Kashahara and Penefsky [35] suggested that the pK_a of a critical amino acid involved in phosphate binding was not titrated in the range of pH values between 6.1 and 8.6. Furthermore, P_i binding to mitochondrial F_1 was considerably reduced by ADP or ATP [30]. According to Ferguson et al. [15], the pK_a for the NBD-Cl-reactive tyrosine residue is 9.5. Moreover, Ting and Wang [39] reported a strong protection by P_i against inactivation of mitochondrial F_1 by NBD-Cl that was largely prevented by low concentrations of ADP. In view of these observations it is conceivable that the NBD-Cl-reactive tyrosine residue in mitochondrial F_1 might be critical for P_i binding.

Studies in *E. coli* coupling factor suggested that the binding site for NBD-Cl is close to or identical with the ADP/ATP site [17]. In the case of $(Na^+ + K^+)$-ATPase from electric eel, the NBD-Cl-reactive residue, whether tyrosine [19] or sulfhydryl [24], was located at the active site.

Recently, Ting and Wang [40] have proposed a catalytic mechanism for synthesis and hydrolysis of ATP by mitochondrial F_1 comprising a topological arrangement of tyrosine, lysine, arginine and glutamine or aspartic acid residues at the active site. The model proposes a direct interaction of the γ-phosphate of ATP with the NBD-Cl-reactive tyrosine residue. The results presented in this report do not fit the model of Ting and Wang. If the NBD-Cl-reactive tyrosine residue in *R. rubrum* F_1 were the binding site of the γ-phosphate of the ATP, a strong protection by ATP should be expected. Conversely, ATP was a poor protector of *R. rubrum* F_1 against inactivation by NBD-Cl.

P_i is an important modulator of the ATPase in membranes of photosynthetic bacteria [41]. The protection by P_i against inactivation of *R. rubrum* F_1 by NBD-Cl can be explained, assuming an allosteric interaction between a regulatory site for phosphate and the NBD-Cl-reactive residue. As yet, the existence of such a regulatory site for P_i in *R. rubrum* F_1 has not been reported.

Alternatively, our results can be rationalized, assuming that *R. rubrum* F_1 holds a P_i domain that is closely related to the catalytic site and contains the NBD-Cl-reactive tyrosine residue. Such a P_i domain should be different from the one that is occupied by the γ-phosphate of the ATP molecule and may play a role in the exit or entrance of P_i during catalysis. Further investigation to test this hypothesis is underway.

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