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CHEMICAL MODIFICATION OF THE β-SUBUNIT ISOLATED FROM A MEMBRANE-BOUND Fo·F_1-ATP SYNTHASE

Modification by 4-chloro-7nitrobenzofurazan does not inhibit restoration of ATP synthesis or hydrolysis

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SUMMARY: The purified, reconstitutively active, β-subunit of the Fo·F_1-ATP synthase of Rhodospirillum rubrum chromatophores was found to bind both 4-chloro-7-nitrobenzofurazan (NBD-Cl) and dicyclohexylcarbodiimide (DCCD). The binding stoichiometry at saturation was 1 mol of either reagent per mol of β. The NBD-modified β-subunit did rebind to the β-less chromatophores and restored all their lost ATP-linked activities as efficiently as the untreated β, whereas the DCCD-modified β-subunit lost completely its capacity to rebind to the depleted chromatophores. These results suggest that the amino acid residue which is modified by NBD-Cl in the isolated β-subunit is not essential for binding and may be also not for activity.

In recent years a large number of chemical modification studies have been carried out on various F_1 preparations in an attempt to identify possible essential amino acid residues and their location on different F_1 subunits (1). Thus, incubation of F_1 complexes from mitochondria, chloroplasts and Escherichia coli vesicles with NBD-Cl (2-4) or DCCD (5-7) resulted in inhibition of their ATPase activity, which correlated with a covalent binding of both inhibitors to the F_1-ATPase. The location of these binding sites on the F_1 subunits was tested by applying labeled inhibitors and searching for the label in the individual subunits obtained after dissociation of the F_1 complexes in sodium dodecyl sulfate. There was usually a fairly specific labeling of the β-subunit, although in most studies some label appeared also in α (1), and in one case the NBD-Cl label appeared primarily mainly in the α-subunit (8).

We have earlier shown that extraction of Rhodospirillum rubrum chromatophores with LiCl removed completely the β-subunit of their Fo·F_1-ATP synthase leaving all the other subunits attached to the membrane (9). The resulting β-less chromatophores lost all their ATP synthesis and hydrolysis activities (9) but retained their light-induced proton uptake (10) and upon reconstitution of the missing β-subunit the ATP-linked activities were restored to more than 80% (9, 11, 12). This removed active β-subunit has been purified to homogeneity (9, 13) and antibodies raised against it were shown to inhibit all ATP-linked activities in R. rubrum chromatophores, RrFo·F_1, RrF_1 as well as in lettuce

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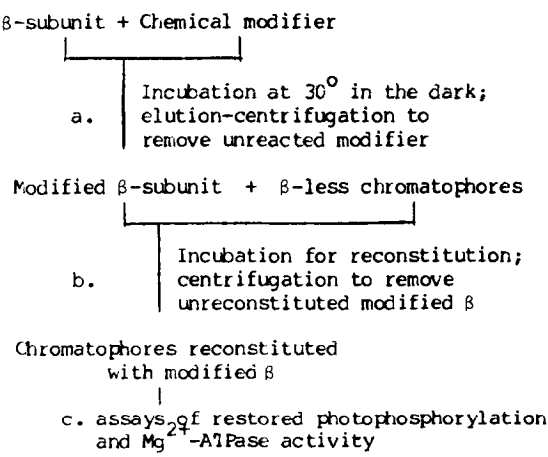
chloroplasts and CF_1 (14). These results clearly indicate that the β-subunit is absolutely necessary for the catalysis itself, but they do not clarify where the catalytic site is located.

Using this unique R. rubrum system we could modify directly the purified actively reconstitutable β-subunit and determine the capacity of such modified β to rebind to the β-less chromatophores and restore their ATP-linked activities. This paper describes the modification of the purified β-subunit with NBD-Cl or DCCD. The two modified β preparations were found to exhibit marked differences in their capacity to rebind to β-less chromatophores and restore their activity.

MATERIALS AND METHODS

Cells, chromatophores and β-subunit. R. rubrum cells were grown as in (9), coupled or β-less chromatophores were prepared according to (13) and the β-subunit was purified and stored as described in (13). Before any further treatment of β the storage buffer was removed by elution-centrifugation on a Sephadex G-50 column (15) equilibrated with the treatment buffer (see below).

Chemical modification. Inactivation and labeling with NBD-Cl was carried out as follows. Chromatophores or the β-subunit in 50 mM Tricine-NaOH, pH 7.6, 20% glycerol, 50 mM NaCl and 2 mM EDTA were treated with various concentrations of NBD-Cl for different times at 30° in the dark. The treated chromatophores were diluted a 100 fold in 50 mM Tricine-NaOH, pH 8.0 and immediately assayed. The modified β-subunit was freed from unreacted NBD-Cl by elution-centrifugation on Sephadex G-50 equilibrated with the reconstitution buffer containing 50 mM Tricine-NaOH, pH 8.0; 4 mM ATP and 20 mM $MgCl_2$. 100 μg protein from each excluded fraction were reconstituted into β-less chromatophores containing 10 μg of bacteriochlorophyll which were immediately assayed for restored activity (see Scheme 1). Experiments testing the binding of (^{14}C) NBD-Cl to the β-subunit were carried out as outlined above and the excluded fraction was either assayed for ^{14}C radioactivity in β or reconstituted into β-less chroma-



Scheme 1. Outline of steps involved in testing for a. binding of the chemical modifiers by the isolated β-subunit; b. reconstitution of the modified β into β-less chromatophores and c. restoration of ATP-linked activities in the chromatophores reconstituted with modified β.

tophores which were assayed for ^{14}C radioactivity as well as for restored activity. Inactivation and labeling with DCCD was carried out in a similar manner, except for the treatment buffer which was 50 mM MES-NaOH, pH 6.0, 20% glycerol, 50 mM NaCl and 2 mM EDTA. Control experiments indicated that the reconstitutive activity of the β-subunit was stable when kept at 30° in both treatment buffers for at least several hours.

Reconstitution and assays. Reconstitution of native or modified β into β-less chromatophores was as in (13) including centrifugation of the reconstituted chromatophores to remove any remaining free β. The reconstituted chromatophores were dissolved in 50 mM Tricine-NaOH, pH 8.0 and assayed for restored photophosphorylation and Mg^{2+}-ATPase activities as described in (13). Protein was determined according to Lowry et al. (16) and bacteriochlorophyll according to Clayton (17). ^{14}C radioactivity counting was according to (15). Binding data of (^{14}C)NBD-Cl or (^{14}C)DCCD to the β-subunit were calculated by using a molecular weight of 50,000 (18).

(^{14}C)NBD-Cl (109 mCi/mmol) and (^{14}C)DCCD (54 mCi/mmol) were purchased from the Commissariat a l'Energie Atomic (CEA, Saclay, France). All other reagents were obtained from commercial sources.

RESULTS AND DISCUSSION

Photophosphorylation as well as Mg^{2+}-ATPase activities of R. rubrum chromatophores were inhibited upon incubation with NBD-Cl at pH 7.5 (Fig. 1). The rate of inactivation followed pseudo first order kinetics and was identical for both ATP-linked activities. Addition of dithiothreitol to the NBD-treated chromatophores restored completely both activities. These effects of NBD-Cl and dithiothreitol on ATP synthesis and hydrolysis by R. rubrum chromatophores are very similar to those reported for the membrane-bound ATPase of submitochondrial particles (2) and for various soluble F_1-ATPases (2-4). In these studies it was suggested that NBD-Cl binds mainly to the β-subunit of the F_1-ATPases. But, in the only case in which the binding of NBD-Cl to isolated F_1-

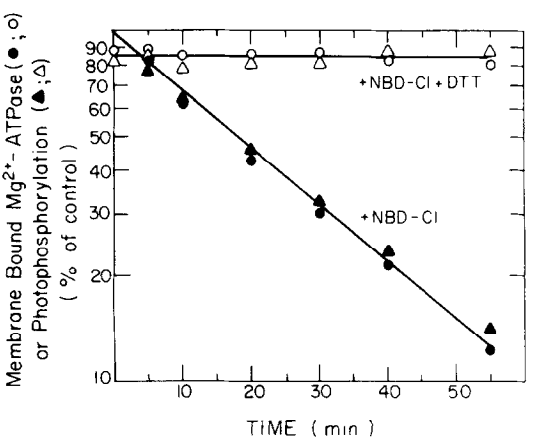


Fig. 1: Kinetics of inactivation of ATP synthesis and hydrolysis in R. rubrum chromatophores by NBD-Cl and its reversibility by dithiothreitol. Coupled chromatophores (0.25 mg bacteriochlorophyll/ml) were treated with 100 μM NBD-Cl for the indicated time intervals as described in Materials and Methods. Aliquot samples were diluted and immediately assayed for Mg^{2+}-ATPase (●,○) or photophosphorylation (▲,△) activity. The dilutions and assays were carried out in the absence (●,▲) or presence (○,△) of 2 mM dithiothreitol (DTT). The control photophosphorylation and Mg^{2+}-ATPase activities were in μmol/h per mg bacteriochlorophyll, 940 and 200 respectively.

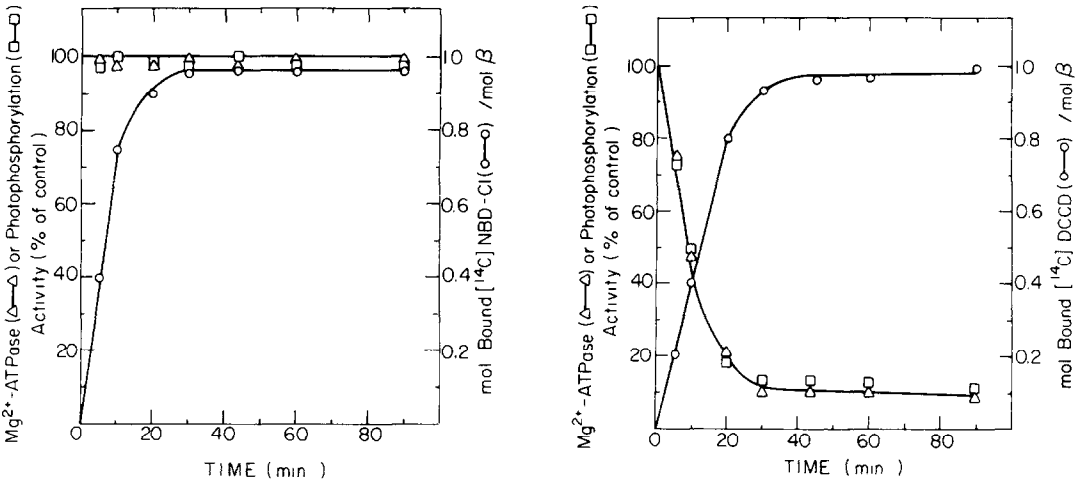


Fig. 2: Time course of incorporation of (^{14}C) NBD-Cl and (^{14}C) DCCD into the purified β-subunit and its effect on the ability of β to reconstitute β-less chromatophores and restore their ATP synthesis and hydrolysis activities. The purified β-subunit (1 mg protein/ml) was treated with NBD-Cl or DCCD as described in Materials and Methods using 300 μM NBD-Cl or (^{14}C) NBD-Cl and 100 μM DCCD or (^{14}C) DCCD. At the indicated time intervals aliquot samples of 100 μg protein from each treatment were freed from the unreacted reagent and assayed for (^{14}C) radioactivity on β or reconstituted into β-less chromatophores which were assayed for restored ATP-linked activities. The control photophosphorylation and Mg^{2+}-ATPase activities restored in β-less chromatophores after their reconstitution with untreated β were, in μmol/h per mg bacteriochlorophyll, 850 and 200 respectively.

subunits has been tested, it was found to bind to both the α and β-subunits (19). These NBD-modified α and β-subunits were, however, not tested for activity.

In the experiments illustrated in Fig. 2 we have examined a. the binding of NBD-Cl and DCCD to the β-subunit that has been isolated from the membrane-bound Fo·F_1-ATPase of R. rubrum chromatophores and b. the capacity of the modified β preparations to reconstitute the β-less chromatophores and restore their ATP-linked activities. Both NBD-Cl and DCCD were found to bind to the isolated, purified β-subunit in a typical saturation curve resulting in a binding stoichiometry of 1 mol of either NBD-Cl or DCCD per mol β (Fig. 2). As has been reported for the F_1-ATPases (2-4, 19), the binding of NBD-Cl to β was accompanied by the appearance of an absorption peak at 380-385 nm which disappeared upon addition of dithiothreitol (not shown).

The two modified β preparations exhibited marked differences in their capacity to restore ATP-linked activities to the β-less chromatophores. The NBD-modified β-subunit was found to restore both photophosphorylation and Mg^{2+}-ATPase activities as efficiently as the untreated β, whereas the binding of DCCD was accompanied by a parallel decrease in the capacity of the modified β-subunit to restore the ATP-linked activities (Fig. 2). The results with DCCD could be due to two possible effects of the

Table 1

Effect of modification of the β-subunit by NBD-Cl and DCCD on its ability to rebind to β-less chromatophores

Subunits added during the periods of reconstitution		Binding of NBD-β or DCCD-β to β-less chromatophores	
First	Second	Total cpm bound to the chromatophores	Ratio of bound modified β
			pmol/mol Bchl
(^{14}C) NBD-β	-	96911	20.3
β	(^{14}C) NBD-β	7962	1.8
(^{14}C) DCCD-β	-	684	0.4
β	(^{14}C) DCCD-β	689	0.4

The purified β-subunit (1 mg protein/ml) was treated with 300 μM (^{14}C) NBD-Cl or 100 μM (^{14}C) DCCD for 1 hr and freed from unreacted reagents as described in Materials and Methods. 100 μg of either untreated β, (^{14}C) NBD-β (5.2×10^5 cpm) or (^{14}C) DCCD-β (2.1×10^5 cpm) were reconstituted into β-less chromatophores at the indicated order, each period of reconstitution being followed by centrifugation as described in Materials and Methods. In the supernatant the binding stoichiometry of (^{14}C) NBD-Cl and (^{14}C) DCCD to β in the remaining non reconstituted β were determined (see text). The reconstituted chromatophore pellet was washed twice in 50 mM Tricine-NaOH containing 200 mM NaCl and its ^{14}C radioactivity measured as described in Materials and Methods. Bchl = Bacteriochlorophyll.

chemical modifier on the reactivity of β: a. the modified β could lose its ability to rebind to the β-less chromatophores and b. the modified β could retain its ability to rebind to the β-less chromatophores but might lose its capacity to restore their ATP-linked activities.

In an attempt to differentiate between these possibilities a direct test of the rebinding of (^{14}C) NBD-β and (^{14}C) DCCD-β to β-less chromatophores was carried out (Table 1). The results clearly demonstrate that more than 18% of the applied (^{14}C) NBD-β as compared to less than 0.3% of the applied (^{14}C) DCCD-β, did rebind to the β-less chromatophores. It should be emphasized that both the (^{14}C)DCCD and the (^{14}C) NBD-Cl did not dissociate from the modified β-subunit during the reconstitution and centrifugation, since there was no decrease in their stoichiometry. Thus, the binding stoichiometry of the applied (^{14}C) NBD-β as compared to the unreconstituted one, which remained in the supernatant after reconstitution and centrifugation (see legend to Table 1), was 1.032 and 1.026 respectively in experiment 1 of Table 1 and 1.032 and 1.011 respectively in experimen 2 of Table 1. For (^{14}C) DCCD-β these numbers were 1.025 and 1.000 in experiment 3 and 1.025 and 1.020 in experiment 4.

The results summarized in Fig. 2 and Table 1 indicate that the DCCD-modified β-subunit lost its ability to rebind to the β-less chromatophores and consequently could not restore any of their ATP-linked activities. On the other hand the NBD-modified β-subunit retained its rebinding capacity and it is, therefore, concluded that the amino acid residue which is modified in the isolated β-subunit by NBD-Cl can not be essential for rebinding. The fact that upon rebinding of NBD-β to β-less chromatophores both their photophosphorylation and Mg^{2+}-ATPase activities were restored could suggest that this amino acid residue is not essential also for activity. This conclusion should, however, await further experimentation, since there is one other possibility that the experiments summarized above did not rule out namely, that the (^{14}C) NBD label, although not dissociating at all during reconstitution, could dissociate during the assays for the various activities.

Our results with DCCD demonstrate the importance of a clear cut differentiation between inhibition of rebinding as compared to inhibition of the capacity to restore activity when testing the effect of various inhibitors on isolated F_1 subunits. Harris and Baltscheffsky (20) have recently reported that reconstitution of an efrapeptin-treated β-subunit into β-less chromatophores has restored only a small fraction of their ATPase activity. As they give no data on the degree of rebinding of the efrapeptin-modified β-subunit it is as yet impossible to decide whether efrapeptin affects the rebinding or the restoration of activity.

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