On the Accessibility of Essential Tyrosines in Isolated and Activated Chloroplast H⁺-ATPase

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The addition of 7-chloro-4-nitrobenzofurazan to isolated and activated CF₅ creates a completely changed binding stoichiometry and subunit-distribution of bound modifier in contrast to the binding pattern in not-activated CF₅. The activation of CF₅ by dithiothreitol and heat results in the accessibility of three additional tyrosines in β-subunits and one additional tyrosine in α-subunits. Binding of pyridoxalphosphate to lysine in the active state suppresses the accessibility of the additional tyrosines, suggesting that PLP inactivates the ATPase by inducing a conformational change. Furthermore, two NBD-molecules are bound to γ-subunits when CF₅ is activated. These two molecules may be bound to sulphydryl groups of cysteines which become accessible to NBD after activation.

Introduction

Isolated chloroplast H⁺-ATPase is a latent ATPase which has to be activated in order to perform ATP-hydrolysis. The subunit stoichiometry of CF₅ is α₃β₃γ₅ε [1]. The larger subunits α and β contain 4 distinct nucleotide binding sites [2], three of which are comprehensively characterized by Bruist and Hammes [3]. The first site (site 1) contains tightly bound ADP which is readily exchangeable with medium nucleotides, the second one (site 2) is a tight MgATP binding site which most probably has structural functions only [4] and site 3 binds nucleotides reversibly under different conditions. The γ-subunit contains four cysteiny1 residues [5] and is involved in the regulation of the catalytic function of CF₅. Two of the cysteiny1 residues form a disulfide bond which can be cleaved by reduction. The resulting sulphydryl groups become accessible to labeling by modifying reagents.

Activation of isolated latent ATPase can be achieved by treatment with heat and dithiothreitol (DTT) [6]. Thioleagagents induce the reduction of the disulfide bond in the γ-subunit [7], whereas heat causes dissociation of the ε-subunit [8, 9].

To understand the catalytic mechanism of any enzyme, it is necessary to identify functional groups which bind the substrates and change their properties during catalysis. ADP and ATP both have at least three points of attachment to the protein: Two of them by ionic interaction of the charged Mg²⁺ and a negatively charged oxygen of the phosphate chain (Pₐ) with the enzyme [10], one by a hydrogen-bridge formed by the hydroxyl residue of tyrosine to the base moiety of the nucleotide molecule. Two different tyrosines proposed to belong to different nucleotide binding sites, could be labeled by covalently bound 2-azido-ADP/ATP [11].

The group-directed reagent 7-chloro-4-nitrobenzofurazan was reported to react with tyrosine residues in enzymes [12–14]. We reported recently [23] that NBD-CI reacts quite specifically with tyrosine residues in CF₅, if a maximum pH of 7.5 is maintained. Furthermore we reported, that isolated nucleotide-depleted CF₅ binds two NBD-molecules to tyrosines with different pseudo-first-order reaction constants, one of them belonging to one α- and the other one to one β-subunit. One NBD/CF₅, however, is sufficient to suppress the activity of the ATPase [15 and this paper]. This paper in hand reports on NBD-binding pattern and -kinetics of isolated and activated CF₅, which are completely different to those of not-activated (latent) one, manifesting large conformational changes of the enzyme during activation.
Materials and Methods

Chloroplast F₁ was isolated and stored as described by Schumann et al. [16] with the exception that no ATP was added to the storage solution. Before use, CF₁ was collected by centrifugation, dissolved in a minimum amount of Tris/EDTA-buffer (50 mM/2 mM), pH 7.5, and passed twice through Penefsky columns [17]. This method was standardly used to get nucleotide-depleted CF₁ according to Feierabend and Schumann [4].

Activation of the ATPase was achieved by incubation of the samples for 4 min at 60 °C in a medium containing 25 mM Tricine pH 8.2, 2 mM EDTA, 10 mM DTT and 40 μM ATP. After 4 min of incubation an aliquot of saturated ammonium sulphate was added to give 50% (precipitation), followed by 5 min on ice, centrifugation, redissolution of the protein in Tris/EDTA-buffer (50 mM/2 mM) pH 7.5, and passing through a Penefsky column equilibrated with the same buffer. The same procedure inclusive incubation in the above described medium, but without DTT, was carried out with not activated controls prior to modification. Ca-ATPase activity of the enzyme was measured in a medium containing 25 mM Tricine pH 8, 5 mM CaCl₂, 5 mM 32P-labeled ATP and 4–10 μg activated CF₁ (final volume 0.5 ml). 32P-labeled ATP was prepared by a method of Avron [18].

Preparation of tyrosine-bound NBD
(O-Tyr-NBD-CF₁)

Reactions with unlabeled NBD-Cl were initiated by addition of NBD (in ethanol, final concentration 100–400 μM, as indicated) to 1.5–3 μM CF₁ in Tris/EDTA-buffer pH 7.5. Binding of NBD was followed spectrophotometrically. The amount of bound NBD was calculated using the extinction coefficient of 11600 M⁻¹ cm⁻¹ (385 nm) [19] for latent CF₁ and 11700 M⁻¹ cm⁻¹ (400 nm) [20] for activated CF₁. Absorbance was usually measured against a reference cuvette containing an equal concentration of NBD-Cl in buffer (in the absence of protein). The absorbance of an equal concentration of protein (in the absence of modifying reagent) was subtracted. The reaction with NBD-Cl was terminated by precipitation of the enzyme with ammonium sulphate. After 10 min on ice, the precipitate was collected by centrifugation, redissolved in 100 μl of Tris/EDTA, pH 7.5, and passed through a Penefsky column.

Binding of 14C-labeled NBD was accomplished in the same way as described for unlabeled NBD. [14C]NBD was purchased from CEA, France (spec. act. 4.81 GBq/mmol). After removing the excess label by centrifugation according to Penefsky, the NBD-O-tyrosyl group had to be stabilized before transferring the protein to the gel. This was achieved by conversion of the nitro group of the NBD into an amino group by chemical reduction with 10 mM sodium dithionite [21]. The subunit distribution was studied in SDS-polyacrylamide gels (17%), after staining with Coomassie blue. The subunit bands of CF₁ were cut out, solved in 30% alkaline H₂O₂ [22] and measured by liquid scintillation counting. The measured amounts of totally bound, labeled NBD-Cl were identical to those measured by the spectrophotometrical method.

Preparation of PLP-modified CF₁

For PLP-modification, the desalted protein was resuspended in 25 mM Tricine-buffer pH 8.5, plus 5 mM MgCl₂. The reaction was started by addition of 5 mM PLP and stopped with NaBH₄ (reduction of the Schiff base) after 15 min. After this procedure excess PLP was removed by the above described method. All reactions were carried out at room temperature and in the dark.

Preparation of lysine-bound NBD
(N-Lys-NBD-CF₁)

N-Lys-NBD-CF₁ was prepared from O-Tyr-NBD-CF₁ (which was cleaned from free NBD) by adding a sufficient amount of Tris-buffer (2 M), pH 9, to increase the pH to 9 as described by Ferguson et al. [19]. The amount of bound NBD in this case was calculated using the absorption coefficient for N-NBD chromophores of 26000 M⁻¹ cm⁻¹ (475 nm).

Results

Binding of NBD-Cl to activated and latent CF₁ and correlation of the inactivation of ATPase to NBD-binding

As we reported in a preceding paper [23], addition of NBD-Cl to isolated and activated CF₁ at pH 7.5 leads to a time-dependent increase of ab-
sorbance at 385 nm, which belongs to a tyrosine-NBD-adduct [19]. Fig. 1 shows the result of simultaneously measured binding of NBD-Cl to isolated, latent CF$_1$ (increase in absorbance at 385 nm) and inactivation of the enzyme (inhibition of ATP-hydrolysis) by the bound NBD. Almost no activity could be measured after 30 min incubation of the latent CF$_1$ with NBD-Cl. At this time 1.6 NBD were bound per CF$_1$. As reported in the preceding paper, inhibition of ATPase activity is completely reversible by addition of dithiothreitol. This was reported earlier for F$_1$ from *E. coli* and BF$_1$ [13, 14]. As a transfer of the NBD-moiety is impossible from lysine (amino group) to DTT (sulfhydryl) and rather unlikely from cysteine (sulfhydryl) to DTT (sulfhydryl), this reaction can be taken as a measure for the amount of bound O-tyr-NBD.

The absorbance spectrum of NBD-CF$_1$ shows a maximum at 410 nm if the enzyme was preactivated, in contradistinction to the not activated enzyme with the usual absorbance maximum between 385–395 nm (Fig. 2). This shift to 410 nm indicates the appearance of additional sulfhydryl group-bound NBD molecules which cause a derivative with an absorbance peak at 420 nm [14]. The observed maximum is clearly due to CF$_1$-bound NBD as it remains bound after repeated washings (precipitation with ammonium sulphate and redissolution) which were terminated by passing the protein solution through a Penefsky column. The lower line in Fig. 2 shows that no NBD remains bound after 30 min incubation with 5 mM DTT followed by precipitation of the protein with ammonium sulphate, redissolution, and passing through a Penefsky column.

Fig. 3 shows the time-dependent inactivation of Ca-ATPase-activity by covalent binding of NBD to activated CF$_1$. The inactivation correlates with the increase in absorbance at 400 nm. After 30 min incubation of the activated enzyme with NBD-Cl, 4 NBD molecules were bound to CF$_1$ and 20% of activity was left. When 5 mM DTT had been added at this time, more than 80% of the activity could be restored. This result corresponds to the fact that almost no bound NBD was found after DTT-treatment (compare Fig. 2, C).
Fig. 3. Time-course of modification of DTT- and heat-activated ATPase at pH 7.5 and simultaneously measured inhibition of Ca-ATPase activity. 2 mg CF₁ were incubated with 100 μM NBD-Cl in ethanol and the time-dependent increase in absorbance followed at 400 nm. At various times aliquots of the mixture were taken and precipitated with ammonium sulphate. After the excess NBD had been removed, ATPase activity was determined. Each rate is calculated from kinetical analysis. 100% activity: 120 μmol [³²P] Pᵢ/mg protein per h.

Fig. 4 shows the inhibition of Ca-ATPase activity as a function of bound NBD/CF₁. As we already have shown [15, 23], one NBD/CF₁ is sufficient for the complete inactivation of the ATPase activity when the enzyme has been modified prior to activation. However, reaction of 5 NBD per CF₁ are needed in case of activated and afterwards modified enzyme.

Distribution of bound ¹⁴C-labeled NBD-Cl to the subunits of CF₁

Using [¹⁴C]NBD as a label and cutting the α, β and γ-bands out of a SDS-gel, we found a completely changed subunit distribution of the bound modifier compared to the binding pattern in not-activated (latent) CF₁ (Table I). In this experiment a total amount of 8.2 mol NBD is bound per mol of activated CF₁ in contradistinction to 2.7 mol NBD in the not activated enzyme. This means that in activated CF₁, 5–6 NBD can bind to places which are not accessible in latent CF₁: one of them in α-, 2–3 in β- and 2 in γ-subunits (Table I, line 3).

Ceccarelli et al. [29] have found that the NBD-tyrosine derivative formed at pH 7.5 was hydrolyzed when the pH was raised to 9. In the contrary to their report, we demonstrated in a preceding paper [23] the time-dependent shift in the absorbance maximum from 385 to 475 nm, when O-tyr-NBD-CF₁ was incubated at pH 9, indicating the intramolecular transfer of NBD from tyrosine-hydroxyls to ε-amino groups of lysines. After this transfer succeeded, the N-lys-NBD-enzyme can be detected by the characteristic fluorescence of the sample. As already shown by Ferguson et al. [19], the excitation maximum of the N-NBD chromophore is at 468 nm and the emission maximum at

![Graph](image-url)

Table I. Quantitative evaluation of ¹⁴C-labeled NBD bound to separated subunits of activated CF₁.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total CF₁</th>
<th>mol[¹⁴C]NBD/mol CF₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α</td>
</tr>
<tr>
<td>Activated</td>
<td>8.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Not activated</td>
<td>2.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Difference</td>
<td>5.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

NBD incubation of isolated latent and heat and DTT-activated CF₁ (1.5 h, 380 μM NBD-Cl, 2 μM CF₁) at pH 7.5 was stopped by precipitation with ammonium sulphate. The protein was collected by centrifugation, redissolved and passed through Penefsky columns. To avoid intramolecular transfers or loss of label by the following procedures, the samples were reduced by 10 mM sodium dithionite (10 min). Other conditions as in “Materials and Methods”.

![Graph](image-url)
522 nm. We found this characteristic fluorescence in α- and β-bands of CF₁ within SDS-gels containing enzyme-bound N-lys-NBD.

If O-tyr-NBD-CF₁ which had previously to modification been activated, was incubated at pH 9 over night (Fig. 5), a peak at 420 nm additional to a shoulder at 475 nm appeared. This observation clearly confirms the involvement of sulfhydryl-bound NBD, which was not shifted to lysines at pH 9 like the tyrosine-bound NBD. From the difference between both spectra (A - B) in Fig. 5 ε = 13000 M⁻¹ cm⁻¹ at 420 nm, ref. 14) 1.8 cysteine-bound NBD molecules per CF₁ could be calculated in the activated sample, whereas 2.1 NBD migrated to lysines in latent CF₁ and 2.9 to lysines in activated CF₁. The value of 1.8 NBD molecules bound to cysteines, corresponds very well with the value of 2 additional NBD-molecules which are bound to γ-subunits of CF₁ (Table I). Thioreductants induce the reduction of the disulfide bond in the γ-subunit [7], which means that two more SH residues become accessible to NBD after activation of the ATPase.

Recently, Ceccarelli et al. [28] published their findings that at least 4 mol of S-Cys-NBD had been formed per mol activated CF₁. These authors have calculated their value from the increase in absorbance at 420 nm using ε for Cys-NBD-chromophores. They failed to subtract the part of absorbance at 390 nm. As shown in Fig. 2 and 5, clearly both forms, S-cys-NBD-CF₁ and O-tyr-NBD-CF₁ have been formed with activated CF₁. However, from the special chemistry of these “Meisenheimer” transitions only the part of NBD bound to tyrosines, is able to be transferred to lysine residues after the shift of the pH value to 9.

**Double-labeling of CF₁ with pyridoxalphosphate and NBD-Cl**

Pyridoxal-5-phosphate (PLP) is known to form a Schiff base (aldimine) with the ε-amino group of lysine. By reduction with NaBH₄ the relatively labile aldime is converted into a stable secondary amine [24]. Treatment of latent [25] and of membrane-bound CF₁ [26] with PLP results in covalent modification of lysine residues causing the inactivity of the enzyme.

In Fig. 6, inhibition of Ca-ATPase activity of the latent enzyme by PLP modification as a function of PLP-concentration is shown. Inactivity usually is reached within 15 min incubation with 10 to 15 mM PLP. Using 5 mM ³H-labeled PLP and 15 min incubation time, 2.5 ± 1 PLP/CF₁ were
bound to ε-amino groups of lysines on the enzyme (not shown).

The amount of bound unlabeled PLP can also be calculated from the maximum in absorbance at 325 nm using the extinction coefficient of 10000 M\(^{-1}\) cm\(^{-1}\) [27]. The two methods give corresponding values.

Our earlier experiments with membrane-bound ATPase have shown, that tritium-labeled PLP binds to α- and β-subunits of CF, and that one binding site in α as well as one in β can be protected by addition of an excess of substrate [26].

The distribution of bound \([14\text{C}]\text{NBD}\) within the subunits of CF, is almost identical in the activated and PLP-modified ATPase and in latent, not modified enzyme (Table II). In both cases 0.8 NBD are bound to α-subunits, 1.2 to β and 1 to γ. This indicates that the binding of PLP either inactivates the enzyme by inducement of a conformational change or that PLP binds very closely to the NBD-modifiable tyrosines, preventing their accessibility to NBD. The last possibility seems to be less probable, as then the stoichiometry of 2.5 PLP/CF, preventing the binding of 4.8 NBD/CF, cannot be explained. Moreover, binding of NBD to γ-subunits is as well reduced to the control level.

In the not-activated enzyme the effect of PLP is less pronounced. However, it affects both, the binding of NBD to β- and to α-subunits (Table II, columns 2 and 3). In both cases 2.5 mol PLP have been measured to be bound to one mol of CF,.

### Table II. Quantitative evaluation of \([14\text{C}]\text{NBD}\) bound to separated subunits of activated and latent pyridoxal-5-phosphate modified ATPase compared to unmodified enzyme.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total CF,</th>
<th>mol[14C]NBD/mol CF,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Subunits</td>
</tr>
<tr>
<td>Activated − PLP</td>
<td>8.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Activated + PLP</td>
<td>3.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Difference</td>
<td>4.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Latent − PLP</td>
<td>3.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Latent + PLP</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Difference</td>
<td>1.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Conditions see Table I and “Materials and Methods”.

### Discussion

It has been known for more than ten years that a single tyrosine residue in different ATPases can be modified by covalent binding of NBD-Cl, and that the modification inactivates the enzyme. Meanwhile the modified tyrosine residue has been identified to be Tyr-β-328 (CF, [28], Tyr-β-311 (mitochondrial ATPase, MF, [21]) and Tyr-β-307 (ATPase of a thermophilic bacterium, TF, [29]). All three are homologuous amino acids within the β-subunits of the different ATPases.

In a preceding paper [23] we described the modification of two tyrosines, one in α- and one in β-subunits when nucleotide-depleted CF, was used. The one in β is the same one that is involved in inactivation of ATPase, if modified. This paper in hand, shows that things become more complicated in case of previously activated ATPase. In this case up to 9 NBD molecules can be found on CF, of which are bound to β-subunits. It was surprising, that the activated enzyme binds up to five NBD until it is totally inactivated. In this state (after about 2 h of incubation with NBD) two of the bound NBD molecules are bound to sulfhydryl groups and 5–6 to tyrosines. There are indications which make us believe that the SH-bound NBD molecules are those found in γ-subunits, as DTT treatment results in the reduction of one disulfide bond in γ, yielding two reactive sulphydryls. But this interpretation has yet to be proved. Nevertheless, two additional \([14\text{C}]\text{NBD}\)-molecules can be found in γ of previously activated CF,.

Each α- and each β-peptide chain of CF, contains one cysteine in the region of the N-terminal end which, however, are not free accessible to SH-modifying reagents, as could be shown using N-ethylmaleimide and the SH-reactive probe 6-acryloyl-2-dimethylaminonaphthalene (Acrylo-dan) [Schumann, personal comm. and ref. 16]. The recently published result of Ceccarelli et al. [28] that labeling of CF, with iodoacetamide prior to the addition of NBD eliminates labeling of the γ-subunit, corroborates our assumption that the additional SH-bound NBD molecules are bound to γ.

When O-tyr-NBD-CF, was incubated at pH 9, a time-dependent shift in the absorbance maximum from 385 to 475 nm could be observed (compare Fig. 5 and ref. [23]). The lysine, to which NBD spe-
cifically migrates under alkaline conditions in MF, is Lys-β-162 (Andrews et al., ref. [30]) and in TF, is Lys-β-164 [31]. These lysines correspond to Lys-β-178 in CF, which is the most conserved lysine within the β-subunits of different ATPases. We are now testing, whether this lysine is the modified one in CF, too. The intramolecular migration of NBD from tyrosine to lysine suggests that either Tyr-β-328 and the specific lysine lie close to each other in the tertiary structure of the native enzyme or a conformational change within the enzyme renders the transfer. The last possibility supposes that a change of the pH value from 7.5 to 9 induces such a conformational change within the protein.

The transfer of NBD from a hydroxyl group of tyrosine to an ε-amino group of lysine happens in α- as well as in β-subunits of CF,. Binding of NBD-C1 to α- and β-subunits can be observed working under conditions avoiding contamination of the enzyme with MgATP. Meanwhile we purified a labeled peptide which belongs to the C-terminal end of the α-chain (amino acids 415–546). This purified and identified peptide contains 4 tyrosines closely together, one of which is labeled with NBD and protected by MgATP. This result will be the theme of a new paper.

The tyrosine residue which has been found to be involved in catalytic activity by covalent labeling with 2-azido-ADP [11] is located in one of the three β-subunits. The presence of three β-subunits per ATPase points to three tyrosine residues which may become accessible to modification by a conformational change within the enzyme. Most possibly, these tyrosines can be labeled by NBD-C1 after the activation procedure, which induces such a conformational change. This view is corroborated by the results of Xue et al. [2] who found 3.5 3H-labeled nucleotides bound to heat- and DTT-activated ATPase.

The surprising finding that up to five NBD are bound to CF, until total inactivity is achieved, whereas one NBD per CF, suffices to suppress the activity when latent CF, is modified, may have the following explanation: Modification of the one accessible tyrosine residue in one of the three β-subunits of latent CF, may prevent the conformational change of the enzyme. After removement of the label by DTT, normal activation is possible (compare ref. [23]). In the case of activated CF, however, 2–3 NBD bind to the three tyrosines in β-subunits (compare Table I) which are involved in the catalytic activity until total inactivity of the enzyme is reached. This means that the β-tyrosine where the NBD is bound to in latent CF,, is not directly involved in the catalytic activity. It is, however, involved in the conformational changes, which lead to activation of the enzyme. This interpretation is supported by the finding that Tyr328 is the modified residue in latent CF, [28]. This tyrosine is neither the one proposed to belong to the catalytic site nor the one which contributes to the non-catalytic binding site labeled with 2-azido-ATP [11]. Sequence studies on the labeled peptide of latent and prior to modification activated ATPase are in progress.

We believe that PLP, in contrast to NBD, induces an inactive conformation of the ATPase as it binds via Mg2+ to an ADP and P1 binding place within a catalytic site of the enzyme thus blocking the whole catalytic cycle [26]. This accounts for the finding that the ATPase binds more than 4 mol PLP/mol CF,. However, protection of one α- and one β-subunit against modification results in full phosphorylating activity [26]. Beyond that it explains the similar binding patterns in the subunits of both, latent and activated PLP-modified CF,.

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