Volume 258, number 2, 309-312 FEB 07902 December 1989

Investigations on nucleotide binding sites of isolated chloroplast ATPase by modification with 7-chloro-4-nitrobenzofurazan

Susanne Bickel-Sandkötter and Petra Strümper

Institut für Biochemie der Pflanzen, Universität Dusseldorf, Universitätsstraße 1, D-4000 Düsseldorf, FRG

Received 26 September 1989

Addition of NBD-Cl to isolated and nucleotide-depleted CF_1 at pH 7 5 leads to binding of 2 NBD molecules on tyrosines of CF_1, one in one of the 3 α-subunits and one in one of the 3 β-subunits MgADP reduces the amount of bound NBD in β, but not in α Modification as a function of time is biphasic, showing an initial rapid and a slow kinetic component MgATP represses the initial rapid phase of binding Incubation of *O*-Tyr-NBD-CF_1 at pH 9 causes a time-dependent shift of the NBD-molecule from tyrosine to lysine The *O*-Tyr-NBD-formation is an obligatory intermediate for specific *N*-Lys-NBD-formation ADP reduces the formation of the lysine-bound NBD drastically

ATP-hydrolysis, ATPase, Chloroplast, Covalent modification, Subunit labeling, NBD

1. INTRODUCTION

Chloroplast H^+-ATPase has a molecular mass of 400 000 [1] and a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. CF_1 is reported to contain 4 distinct nucleotide binding sites [2], a value which often is extrapolated to 6 for the maintenance of symmetry (3 pairs of α/β). Three of the binding sites are well 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 Mg-ATP-binding site and site 3 binds nucleotides reversibly under different conditions. Catalytic sites as well as 'tight' binding sites are found to be located at the β-subunits or at interfaces of the α- and β-polypeptide chains [2].

The actual substrate used by the ATPase in the hydrolysis direction is the β,γ-Δ-Mg-ATP chelate complex [4]. ATP and ADP have at least 3 points of attachment to the protein. The first one by ionic interaction of the chelated Mg^{2+} to a negatively charged carboxyl group of the protein. The second bond is maintained by ionic interaction of the negatively charged P_α of the nucleotide molecule [4] to a positively charged amino group of the enzyme. This group most probably is contributed by an ε-amino group of a lysine [5]. The third point of interaction is an H-bridge formed by the hydroxyl residue of tyrosine to the base N^1 may exist [6].

Correspondence address S. Bickel-Sandkotter, Institut fur Biochemie der Pflanzen, Universitat Dusseldorf, Universitatsstraße 1, D-4000 Dusseldorf, FRG

Abbreviations NBD-Cl (NBD), 7-chloro-4-nitrobenzofurazan, DTT, dithiothreitol

The H^+-ATPases from a variety of sources are inactivated by the tyrosyl group-directed reagent NBD-Cl which forms a Meisenheimer complex on C^4 with the tyrosyl oxygen atom [7]. Modification of a single tyrosine residue per chloroplast F_1 is sufficient to inactivate the enzyme completely [8,9].

In 1975 it was reported that almost exclusively the β-subunits of CF_1 were labeled by NBD-Cl [10]. Our work shows that under tyrosine-modifying conditions two NBD molecules are bound per CF_1, the first in one of the three α-subunits and the second in one of the three β-subunits. Furthermore we investigated the transfer of NBD from tyrosine to lysine on chloroplast F_1. This transfer has been reported in detail by Ferguson et al. [11] for mitochondrial ATPase. The results are discussed in context with different types of nucleotide binding sites on isolated CF_1.

2. MATERIALS AND METHODS

Chloroplast F_1 was isolated and stored as described by Schumann et al [12] Before use, CF_1 was collected by centrifugation, dissolved in a minimum amount of Tris/EDTA-buffer (50 mM/2 mM), pH 7 5, and passed through a Penefsky column [13]

Preparation of tyrosine-bound NBD (O-Tyr-NBD-CF_1)

Reactions with unlabeled NBD-Cl were initiated by addition of NBD-Cl (in ethanol, final concentration 200 μM if not otherwise indicated) to 1 5-3 μM CF_1 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 11 600 M^{-1} cm^{-1} (385 nm) [11] Absorbance at 385 nm 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 CF_1 (in the absence of NBD-Cl) was subtracted The reaction with NBD-Cl was terminated by precipitation of the enzyme with ammonium sulphate After 10 min on ice, the precip-

Published by Elsevier Science Publishers B V. (Biomedical Division)
00145793/89/$3 50

itate was collected by centrifugation, redissolved in 100 μl of Tris/EDTA, pH 7 5, and passed through a Penefsky column

Ca-ATPase activity of the modified enzyme was measured after trypsin-activation as described by Vambutas and Racker [14] in a medium containing 20 mM Tris, pH 8, 5 mM $CaCl_2$, 5 mM ^{32}P-labeled ATP and 10 μg modified CF_1 (final volume 0 5 ml)

^{32}P-Labeled ATP was prepared by a method of Avron [15]

Binding of ^{14}C-labeled NBD-Cl was accomplished in the same way as described for unlabeled NBD [^{14}C]NBD was purchased from CEA, France (sp act 4 81 GBq/mmol). After removing excess label by centrifugation according to Penefsky [13], 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 sodium dithionite [17] The subunit distribution of the label was studied in SDS-polyacrylamide gels (gradient gel, 17 5–22 5% polyacrylamide), after staining with Coomassie blue The subunit bands of CF_1 were cut out, solved in 30% alkaline H_2O_2 [18] and measured by liquid scintillation counting

Preparation of lysine-bound NBD (N-Lys-NBD-CF_1)

N-Lys-NBD-CF_1 was prepared from *O*-Tyr-NBD-CF_1 (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 [11] The transfer of the NBD group could be followed by observing the shift in absorbance spectrum 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) In other cases (if indicated) desalted CF_1 was redissolved in Tris/EDTA, free NBD-Cl was added and the reaction was followed spectrophotometrically at 475 nm

3. RESULTS AND DISCUSSION

Addition of NBD-Cl to isolated CF_1 at pH 7.5 leads to inactivation of CF_1 which is accompanied by formation of an *O*-Tyr-NBD chromophore with an absorbance maximum at 385 nm. Since phenolic hydroxyl groups react with NBD-Cl to give a derivative with an absorbance peak at 385 nm [11] we can conclude that NBD-Cl at pH 7.5 reacts predominantly with the hydroxyl group of a tyrosyl residue in CF_1. In fig.1, inhibition of Ca-ATPase activity of isolated CF_1 by addition of NBD-Cl at pH 7.5 is shown. In this experiment we observed 85% inhibition within 30 min. As reported earlier for F_1 from *E. coli* [19] and BF_1 [20], inhibition of ATPase activity is completely reversible by addition of DTT. As lysine-bound NBD is not affected by thiol reagents like DTT, this reaction can be taken as a measure for the amount of bound *O*-Tyr-NBD. Using the extinction coefficient given by Ferguson et al. (see section 2) and a molecular mass of 400000 for CF_1, we have calculated a CF_1/bound NBD ratio of 1 (pH 7.5) under conditions where the enzyme has been completely inhibited.

When *O*-Tyr-NBD-CF_1 was incubated at pH 9.0, a time-dependent shift in the absorbance maximum from 385 to 475 nm was observed. Under the employed conditions (nucleotide-depleted CF_1), the shift was complete after 80 min (fig.2). Precipitation with ammonium sulphate and centrifugation-elution of the protein/NBD solution (Penefsky [13]) showed that the NBD completely remained on the enzyme. In contrast

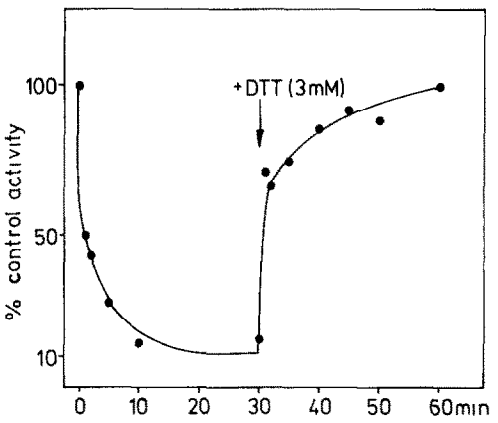

Fig.1 Inhibition of Ca-ATPase activity by modification of CF_1 with NBD-Cl at pH 7 5 2 μM CF_1 was incubated with 80 μM NBD-Cl Other conditions, see section 2 100% activity = 150 μmol P_i/mg protein per h

to the *O*-Tyr-NBD-form, the *N*-Lys-NBD-CF_1 is stable and can be stored as an ammonium sulphate precipitate for more than one month.

The formation of primary amine products occurs at slightly alkaline pH (pH>7.5). The time-course as a function of pH is shown in fig.3. At pH 7.5, 0.2 mol NBD/mol enzyme were bound in the amine-form after 60 min, at pH 8, 1.2 mol/mol, whereas at pH 9 after the same time 2.8 NBD/CF_1 can be calculated. If tyrosine residues should be modified, a pH of 7.5 or less should be strongly kept.

The slopes in fig.3 exhibit a lag-phase which disappears after preincubation of the enzyme with NBD-Cl at pH 7.5 (fig.4, control), showing that *O*-Tyr-NBD formation on CF_1 is an obligatory intermediate for specific *N*-Lys-NBD formation. A similar result has

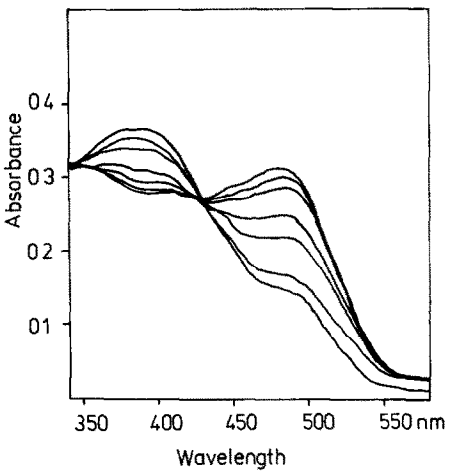

Fig.2. Transfer of the NBD group from a tyrosyl oxygen to a lysyl nitrogen of isolated CF_1, followed by observing the shift in absorbance spectrum 7 5 μM of desalted *O*-Tyr-NBD-CF_1 was dissolved in 40 mM Tris/2 mM EDTA, pH 7 5 Spectra were taken before and 0, 10, 30, 50, 70, and 80 min after transfer to pH 9

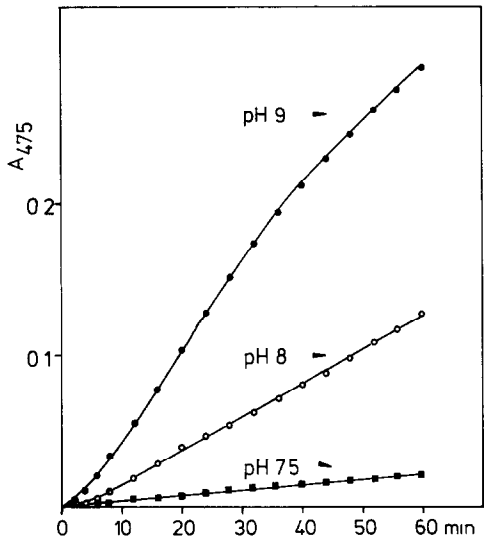

Fig 3. Binding of NBD-Cl to lysine residues of CF_1, measured by the increase in absorbance at 475 nm at 3 different pH values Conditions, see section 2

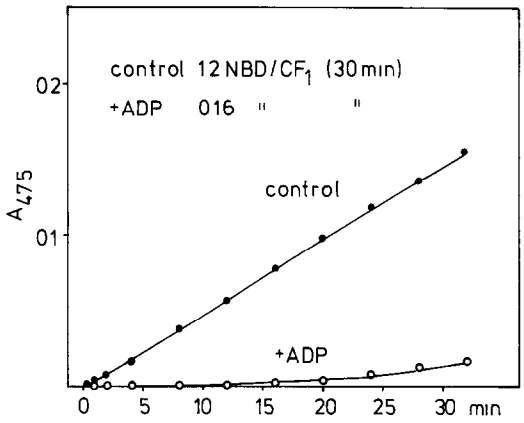

Fig.4 Transfer of the NBD group from a tyrosyl to a lysyl group, followed by the increase in absorbance at 475 nm after a pH shift from 7.5 to 9, in absence (control) or in presence of 2 mM ADP and 10 mM P_i Preincubation time was 40 min

been reported for modification of lysozyme with NBD-Cl [16]. The lysine where NBD is transferred to, belongs to a reversible nucleotide binding site, which is protected against modification by ADP. Addition of excess ADP (in the presence of Mg^{2+} and P_i) to a preparation of *O*-Tyr-NBD before the pH value of the medium is shifted to pH 9, leads to depression of formation of *N*-Lys-NBD, as shown in fig.4. After 30 min, not more than 0.16 mol NBD were bound to lysine per mol of CF_1 in presence of ADP, whereas 1.2 mol/mol CF_1 were bound in the control.

In a previous paper we reported, that 2 mol NBD/mol CF_1 could be found on tyrosine residues if the nucleotide-depleted enzyme was used [9]. Modification of CF_1 at pH 7.5 as a function of time is biphasic, indicating that at least two different tyrosines per CF_1 bind NBD-Cl. The first NBD binds with a pseudo-first-order rate of 0.2 min^{-1}, the second one with 0.06 min^{-1}. Addition of excess ADP suppresses the second slow phase of binding and addition of excess Mg-ATP suppresses the first fast phase of binding, indicating two different sites of NBD-interaction on CF_1 [9].

In table 1 Ca-ATPase activity of isolated CF_1 pretreated at pH 7.5 with NBD-Cl plus/minus nucleotides is shown. Presence of Mg-ATP during the incubation reduces the inhibition only slightly, after 20 min 78% of inhibition could be observed. Presence of ADP, however, led to protection of ATPase against inactivation.

From these results we conclude that the fast reachable site to which NBD is bound may be identical to the tight Mg-ATP-binding site which has no catalytic function (site 2, Bruist and Hammes [3]). Feierabend and Schumann [21] found that this site has structural functions only. This would explain why Mg-ATP is not able to protect the enzyme against loss of activity. The second tyrosine which can be protected by ADP against NBD binding may be localized within or near a catalytically active site.

Using [^{14}C]NBD-Cl we found that the latter (slow) NBD is bound to a β-subunit of CF_1 whereas the fast binding NBD interacts with the α-subunit (table 2). In table 2 furthermore is shown that Mg-ADP is able to decrease the label in the β-subunits, but not in α (line 3). ADP without Mg^{2+} is not able to decrease the amount of bound NBD (line 2).

Our results lead to the conclusion that the NBD-binding tyrosine on the β-subunit may belong to site 1, the one which according to Bruist and Hammes [22] is a regulatory one but with regard to Wu and Boyer [23] has catalytic activity. As site 3 binds ADP in absence of divalent cations too, NBD binding to this site should be suppressed in the presence of ADP without $MgCl_2$.

Reaction of 1 NBD with a tyrosine on CF_1 which prevented binding of nucleotides to the catalytic site and abolished ATPase activity, was already reported by Cantley and Hammes in 1975 [8]. The authors determined 1.6 NBD/CF_1 (pH 8) using an extinction coefficient of 10700 $M^{-1} \cdot cm^{-1}$ at 400 nm. They further assumed 3 NBD-reactive β-tyrosines according to the subunit-stoichiometry of β_3 [24]. Deters et al. [10] have reported that ^{3}H-labeled NBD was bound exclusively to the β-subunits of CF_1. This difference to our results may be explainable by the different experimental conditions. The authors used CF_1 which was not depleted from nucleotides, and they worked at pH 8 using very long incubation times. During the preparation of this work Ceccarelli et al. [25] published results about modification of isolated CF_1 with NBD-Cl. They found 1.2 mol *O*-Tyr-NBD/mol CF_1 bound to β-subunits and that Tyr-β-328 which is homologous to Tyr-β-311

Table 1

Ca-ATPase activity of isolated CF_1 pretreated with NBD-Cl at pH 7 5 ± nucleotides

Incubation time (min)	μmol P_i/mg CF_1 per h					
	control	%	+ ATP	%	+ ADP	%
0	447 3	100	524 2	100	549 0	100
2	117 6	26	208 5	40	355 5	75
10	56 1	13	169 0	32	341 5	57
20	48 3	11	114 3	22	353 0	64

CF_1 (3 μM) was pretreated with 80 μM NBD-Cl at pH 7.5 in presence or absence of the indicated nucleotides which were 2 mM Experimental conditions, see section 2

Table 2

Quantitative evaluation of ^{14}C-labeled NBD bound to separated subunits of CF_1

Conditions	mol [^{14}C]NBD/mol CF_1		
	total CF_1	subunits	
		α	β
control	1 7	0 8	0 9
+ 2 mM ADP	1 6	0 8	0 8
+ 2 mM ADP + 5 mM $MgCl_2$	1 1	0 8	0 3

500 μg nucleotide-depleted CF_1 was incubated at pH 7 5 with 400 μM ^{14}C-labeled NBD-Cl for 1 h Experimental conditions are described in section 2

(MF_1) is that residue which reacts with NBD-Cl at pH 7.5. This tyrosine is neither the one proposed to belong to the catalytic site (identified by covalently bound 2-azido-ADP, derived from 2-azido-ATP) nor the one which contributes to the non-catalytic binding site [26]. Ceccarelli et al. worked with CF_1 in presence of 1 mM ATP; this explains that they found ond NBD/CF_1 (only the one bound to β-subunit). We are now employing the positions of the NBD-binding tyrosines under nucleotide-depleted conditions.

Acknowledgements This work was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 189) The authors thank Dr Sigrid Kleefeld for reading the manuscript

REFERENCES

[1] Moroney, J V, Lopresti, L , McEwen, B F , McCarty, R E and Hammes, G G. (1983) FEBS Lett 158, 58–62
[2] Xue, Z , Zhou, J M , Melese, T , Cross, R L and Boyer, P D (1987) Biochemistry 26, 3749–3753
[3] Bruist, M F and Hammes, G G (1981) Biochemistry 20, 6298–6305
[4] Bickel-Sandkotter, S (1985) Biochim Biophys Acta 809, 117–124
[5] Bickel-Sandkotter, S and Gokus, M (1988) Biochim Biophys Acta 974, 30–35
[6] Vallejos, R.H (1981) in Energy Coupling in Photosynthesis (Selman, B R and Selman-Reimer, S eds) pp 129–139, Elsevier, Amsterdam
[7] Sutton, R and Ferguson, S J (1984) Eur J. Biochem 142, 387–392
[8] Cantley, L C and Hammes, G G (1975) Biochemistry 14, 2968–2975
[9] Bickel-Sandkotter, S and Strumper, P (1989) in· Proceedings of the VIIIth International Congress on Photosynthesis, Stockholm, in press
[10] Deters, D W , Racker, E , Nelson, N and Nelson, H (1975) J Biol Chem 250, 1041–1047
[11] Ferguson, S J , Lloyd, W J and Radda, G K (1975) Eur J Biochem 54, 127–133
[12] Schumann, J , Richter, M L and McCarty, R E (1985) J Biol Chem 260, 11817–11823
[13] Penefsky, H S (1979) Methods Enzymol 91, 486–493
[14] Vambutas, V K and Racker, E (1985) J Biol Chem 240, 2660–2675
[15] Avron, M (1961) Anal Biochem 2, 535–543
[16] Aboderin, A A , Boedefeld, E and Luisi, P.L (1973) Biochim Biophys Acta 328, 20–30
[17] Andrews, W W , Hill, F C and Allison, W.S (1984) J Biol Chem. 259, 8219–8225
[18] Goodman, D and Matzura, H (1971) Anal Biochem 42, 481–486
[19] Lunardi, J , Satre, M , Bof, M and Vignais, V (1979) Biochemistry 18, 5310–5316
[20] Ferguson, S J , Lloyd, W J , Lyons, M H and Radda, G K (1975) Eur J Biochem 54, 117–126
[21] Feierabend, B and Schumann, J (1988) Biochim Biophys Acta 932, 146–152
[22] Bruist, M F and Hammes, G G (1982) Biochemistry 21, 3370–3377
[23] Wu, D and Boyer, P D (1986) Biochemistry 25, 3390–3396
[24] Snyder, B and Hammes, G G (1985) Biochemistry 24, 2324–2331
[25] Ceccarelli, E A , Verburg, J G , Zhou, S and Allison, W (1989) Arch Biochem Biophys 272, 400–411
[26] Xue, Z , Miller, C G , Zhou, J M and Boyer, P D (1987) FEBS Lett 223, 391–394