7-Chloro-4-nitrobenzofurazan Inactivates Chloroplast H^+-ATPase by Modification of Different Tyrosines, Depending on the Presence of ATP

Susanne Bickel-Sandkötter and Kordula Esser

Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, D-40225 Düsseldorf, Bundesrepublik Deutschland

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The addition of 7-chloro-4-nitrobenzofurazan (NBD) to isolated CF_1 at pH 7.5 leads to one tyrosine-bound NBD molecule per CF_1 in one of the three β-subunits, concomittantly with the inhibition of the ATPase activity. Addition of ADP prior to NBD-incubation protected the ATPase activity and reduced binding of NBD to β-subunits. The addition of MgATP prior to modification did not result in protection against modification of the β-subunit as well as preservation of activity. Cleavage of the NBD-labelled subunits with cyanogen bromide, followed by analysis of the labelled peptides, led to detection of a ^{14}C-labelled peptide of 7 kDa in both cases (± ATP-preincubation). Sequence analysis of this peptide showed that in ATP-incubated CF_1, tyrβ328 was modified with NBD-Cl, whereas the ATP free sample contained no NBD bound to this tyrosine. Further digestion of the labelled peptide with protease V8 *(Staphylococcus aureus)* followed by sequence analysis of the radioactive labelled peptide, led to the detection of β-tyr362 as the modified amino acid in case of ATP-free CF_1. Both tyrosines are closely related to a proposed nucleotide binding region of β.

Introduction

The F_0F_1-ATPases are strictly regulated enzymes which couple transmembrane protontransport to ATP-synthesis. The isolated F_1-part of chloroplast ATPase requires activation in order to hydrolyze ATP. The subunit stoichiometry of the enzyme is $\alpha_3\beta_3\gamma\delta\varepsilon$ (McCarty and Moroney, 1985). The larger subunits, α and β, contain 4 distinct nucleotide binding sites (Xue *et al.*, 1987b), three of which are well characterized by Bruist and Hammes (1981). 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 (Feierabend and Schumann, 1988), and site 3 binds nucleotides reversibly under different conditions.

Although the F_1 moiety is the most studied part of the F_1F_0-ATPases, and cristallization and X-ray analysis has been successful realized by Thomas *et al.* (1992); the mechanism of its catalytic activity is not yet fully understood. Recently, a model derived from the structure of the adenylate kinase has been worked out to represent the active part of the ATPases (Duncan *et al.*, 1986).

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. Labelling experiments resulted in detection of several amino acid residues outside the region described by the adenylate kinase. Thus two different tyrosines, proposed to belong to different nucleotide binding sites, could be labelled by covalently bound 2-azido-ADP/ATP (Xue *et al.*, 1987a). Cross *et al.* (1987) introduced a model, which includes these residues.

The group-directed reagent 7-chloro-4-nitrobenzofurazan was reported to react with tyrosine residues in enzymes (Sutton and Ferguson, 1984; Lunardi *et al.*, 1979; Ferguson *et al.*, 1975). We reported recently (Bickel-Sandkötter and Strümper, 1989a, b) that NBD-Cl reacts quite specifically with tyrosine residues in CF_1, if a maximum pH of 7.5 is maintained. Furthermore we reported, that isolated nucleotide-depleted CF_1 binds two NBD-molecules to tyrosines with different pseudo-first-

Abbreviations: CF_1, chloroplast coupling factor 1 (ATPase); DTT, Dithiothreitol; NBD-Cl (NBD), 7-Chloro-4-nitrobenzofurazan; NEM, N-ethylmaleimide; P_i, inorganic phosphate; SDS, sodium dodecylsulfate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; Tris-buffer, tris-hydroxymethyl aminomethane.

Reprint requests to Dr. S. Bickel-Sandkötter, Telefax 0211-311-3706.

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order reaction constants, one of them belonging to one α- and the other one to one β-subunit. One NBD/CF_1 (in β-subunits), however, is sufficient to suppress the activity of the ATPase (Bickel-Sandkötter and Strümper, 1989a, b; Bickel-Sandkötter *et al.*, 1991; Ceccarelli *et al.*, 1989). The paper in hand reports on the localization of the modified tyrosines within the primary structure of the β-subunits. The results will be discussed in context with the existing model of the active site of CF_1.

Materials and Methods

Chloroplast F_1 was isolated and stored as described by Schumann *et al.* (1985). 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 twice through Sephadex G 50 columns according to Penefsky (1977). This method was routinely used to obtain CF_1 free of exchangeable nucleotides according to Feierabend and Schumann (1988).

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 mM EDTA, 10 mM DTT and 40 mM ATP. After 4 min of incubation the solution was diluted with Tris-buffer to 5 mM ATP. Ca-ATPase activity of the enzyme was measured in a medium containing 25 mM Tris pH 8, 5 mM $CaCl_2$, 5 mM ^{32}P-labelled ATP and 4–10 µg activated CF_1 (final volume 0.5 ml). [γ^{32}P] ATP, specific activity 37 MBq/ml was purchased by Amersham Buchler, Braunschweig.

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

Reactions with unlabelled NBD-Cl were initiated by addition of NBD (in ethanol, final concentration as indicated) to 1.5–3 µM CF_1 in Tris/EDTA-buffer pH 7.5. The reaction with NBD-Cl was terminated by precipitation of the enzyme with ammonium sulphate (final amount 50%). 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 Sephadex G-50 column by centrifugation. To avoid a transfer of the NBD-moiety from phenolic hydroxyl groups of tyrosines to sulfhydryl groups of DTT, the washed and redissolved protein was stirred for 5 min at room temperature in presence of 10 mM (final concentration) sodium dithionite. This procedure leads to conversion of the nitro group (O-tyr-ABD-CF_1) of the NBD into an amino group by chemical reduction (Andrews *et al.*, 1984).

Binding of ^{14}C-labelled NBD was accomplished in the same way as described for unlabelled NBD. [^{14}C]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 by addition of sodium dithionite as described above. The amount of NBD bound per subunit was studied in SDS-polyacrylamide gels (17%), after staining it with Coomassie blue. The subunit bands of CF_1 were cut out, solved in 30% alkaline H_2O_2 (Goodman and Matzura, 1971) and measured by liquid scintillation counting.

Separation of subunits of the labelled ATPase

For cleavage, the β-subunits of the [^{14}C]NBD-labelled CF_1 were separated by a preparative SDS-polyacrylamide gel (gradient gel, 10.5–17.5%). The gel was then stained by plunging it into icecold potassium chloride (0.25 M) for a few minutes according to Hager and Burgess (1980) and washing it with distilled water. After cutting out the gel, the band of the β-subunit was cut into very small pieces, filled into sterilized glass vessels and covered with elution buffer, containing 50 mM Tris/HCl pH 8, 150 mM NaCl, 0.1 mM EDTA, 0.1% (mass/vol) SDS and 0.5% (mass/vol) β-mercaptoethanol, and shaken overnight at room temperature (Higgins and Dahmus, 1979). The eluted subunit was precipitated with acetone (80%, by vol.) at −20 °C for three days, collected by centrifugation (10 min, 4 °C, 10,000 rpm, Beckmann-centrifuge, model J2–21). The pellets were dried under vacuum.

Cleavage of labelled subunits with cyanogen bromide and protease from Staphylococcus aureus

For cleavage with cyanogen bromide, the samples were resuspended in Tris/EDTA (50 mM/2 mM) pH 7.5, containing 0.1% SDS, and diluted to 1–2 µg of protein/µl. Formic acid was added to a final amount of 70% (by vol.), and finally 1% (mass/vol) cyanogen bromide was added. After incubation at 37 °C for 3 h, the cleavage procedure

was stopped by addition of a ten-fold volume of icecold water. The cleavage was not complete under the employed conditions, but the peptide pattern was reproducible. The samples were lyophilized, resolved in a small amount of Tris/EDTA containing 1% SDS and mixed with the same volume of buffer, containing 0.1 M Na_2CO_3, 10% Saccharose, 50 mM dithiothreitol and 2.5% SDS and then transferred to SDS-polyacrylamide gels (17% acrylamide).

For digestion with *Staphylococcus aureus* protease (strain V 8), the samples were resuspended in sodium phosphate buffer (20 mM) pH 8, containing 0.05% SDS, and diluted to 1–2.5 µg of protein per µl and V 8 protease was added to a final ratio (protease:CF_1) of 1:20. Incubation at 37 °C followed for 22 h. The digestion procedure was stopped by addition of the same volume of buffer containing 0.1 M Na_2CO_3, 10% Saccharose, 50 mM dithiothreitol and 2.5% SDS, and boiled for 5 min. Separation followed on SDS-polyacrylamide gels (gradient gel, 15–20%).

Determination of radioactivity and sequence analysis

After separation, the peptide bands were Coomassie stained and the radioactivity of the peptides was determined by cutting the bands out of the gel, as described above. The cleaved peptides of a parallel sample were blotted on polyvinylidendifluoride-membrane (Millipore, Eschborn), the identified (by comparison to the ^{14}C-labelled gel) bands were cut out of the membrane and transferred to a gas phase sequencer for automatic amino acid sequence analysis. Sequence analysis was performed by Dr. H. E. Meyer, Ruhr-Universität Bochum and by Dr. R. Schmid, Universität Osnabrück.

Protein determinations were performed either according to Bensadoun and Weinstein (1976) (samples containing SDS) or to Lowry *et al.* (1951).

Results

Inhibition of ATPase activity by NBD and protection against inhibition

Ca-ATPase becomes inactivated by pretreatment of isolated CF_1 with NBD-Cl (Bickel-Sandkötter and Strümper, 1989a, b; Bickel-Sandkötter *et al.*, 1991). This is once more shown in Fig. 1: it

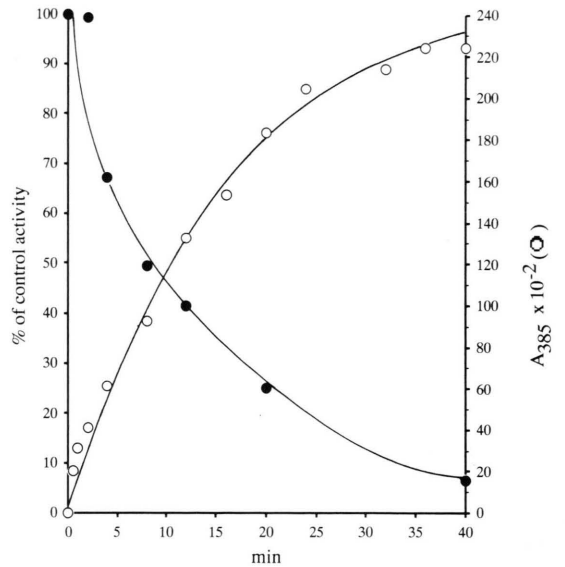

Fig. 1. Binding of NBD-Cl to tyrosine residues at latent, ATP + Mg^{2+} (final concentrations 2 mM/10 mM) incubated CF_1, measured by the increase in absorbance at 385 nm, and simultaneously measured inhibition of Ca-ATPase activity. 440 nmol NBD-Cl in ethanol have been added to 1.5 nmol of CF_1. The increase in absorbance at 385 nm was measured as described earlier (Bikkel-Sandkötter *et al.*, 1991). At various times aliquots of the mixture were taken and precipitated with ammonium sulphate. After the excess NBD had been removed; NBD was reduced with dithionite (see "Methods"), the enzyme was activated and the ATPase activity was determined. 100% activity: 280 µmol $[^{32}P]P_i$/mg protein per h.

shows the results of simultaneously measured binding of NBD-Cl to tyrosine residues of isolated, latent CF_1 (increase in absorbance at 385 nm according to Ferguson *et al.*, 1975) and concomittant inactivation of the ATPase activity. In contrast to earlier experiments, this experiment was performed in presence of 2 mM ATP, which prevents binding of NBD to α-subunits (compare Table II and Fig. 3). After 40 min of incubation, 10% of the control activity is left. At this time 1.3 mol NBD were bound per mol of CF_1. The amount of bound NBD in this experiment was calculated using the extinction coefficient of 11,600 $M^{-1}cm^{-1}$ (Ferguson *et al.*, 1975). This way to determine the ratio of NBD/CF_1 yielded in corresponding values to those recieved *via* measurement of bound $[^{14}C]$NBD/CF_1 (compare f. i. Table II, line 5).

As activation of the enzyme and determination of activity was achieved at pH 8, and increasing

Table I. Ca-ATPase activity of isolated CF_1 pretreated with NBD-Cl at pH 7.5 in presence of different substrates; protective effect of ADP.

Conditions	μmol $[^{32}P]P_i$/mg CF_1 per h	% of control	% protection
Control	596	100	–
100 μM NBD	132	22	0
100 μM NBD + 10 mM $MgCl_2$	220	36.9	14.9
100 μM NBD + 10 mM P_i	244	40.9	18.9
100 μM NBD + 10 mM P_i + 10 mM $MgCl_2$	233	39.1	17.1
Control + 5 mM $MgCl_2$ + 5 mM ADP + 5 mM P_i	542	100	–
150 μM NBD + 5 mM $MgCl_2$	84	15.5	–
150 μM NBD + 5 mM $MgCl_2$ + 5 mM ADP + 5 mM P_i	314	57.9	42.4
Control + 5 mM $MgCl_2$ + PEP + PK	436	100	–
100 μM NBD + 5 mM $MgCl_2$ + PEP + PK	216	49.5	–
100 μM NBD + 5 mM $MgCl_2$ + 5 mM ATP + PEP + PK	233	53.4	3.9

CF_1 (3 μM) was pretreated with NBD-Cl at pH 7.5 in presence of the indicated substances for 20 min. In presence of ATP, the assay contained 5 mM phosphoenolpyruvate and 50 U/ml saltfree pyruvate-kinase, in order to avoid contaminations with ADP.

the pH-value of the medium results in intramolecular transfer of the bound NBD (Bickel-Sandkötter and Strümper, 1989a), the tyrosine bound NBD (O-tyr-NBD) has been standardly reduced to O-tyr-ABD *via* incubation for 10 min in sodium dithionite. The addition of dithionite to a final amount of 10 mM gave no reactivation of the enzyme and no substancial loss of label. Our experiments were usually performed in Tricine pH 7.5. Andrews *et al.* (1984) reported about reactivation of bovine heart mitochondrial ATPase of 30% at pH 7 (MOPS) and 58% at pH 8 (HEPES). We have no explanation for this discrepancy, except for the different enzyme studied.

The protective effect of different substrates on latent ATPase is shown in Table I. High concentrations of $MgCl_2$ have a small protective effect, which may be due to a higher portion of intact ATPases. The same level of protection can be reached by addition of inorganic phosphate, but no additive effect occurs in presence of Mg^{2+} and P_i (Table I, line 5).

Upon addition of ADP together with P_i and Mg^{2+}, we observed about 50% protection against inactivation by NBD-Cl in this experiment.

Tyrosine β328 has been reported by Ceccarelli *et al.* (1989) to be the NBD-modified amino acid. Tyrβ311 in MF_1 (equivalent to tyr328 of CF_1) could be labelled also by 8-Azido-ADP (Wagenvoord *et al.*, 1981) and is assumed to lie within the nucleotide binding region of β. For this reason binding of ATP to the enzyme was expected to protect this region against modification. In a former paper (Bickel-Sandkötter and Strümper, 1989a), we described a protective effect of ATP of 10–20%. If the experiments were carried out in presence of an ATP-regenerating system to avoid contaminations with ADP, no protection was observed (Table I, line 11). The following experiments were done to check the reason for the inability of ATP to protect the nucleotide binding site against modification with NBD.

Binding of NBD to different ATPase subunits

Using $[^{14}C]$NBD-Cl under conditions of total inhibition of the enzyme (compare Fig. 1), we found different patterns of labelled NBD within the subunits of the enzyme, depending on the experimental conditions (Table II):

Latent ATPase binds 2–3 mol NBD/mol CF_1. To ensure that the NBD was not bound to the only cysteine within one α- or β-subunit, preincubation with the sulfhydryl-modifying reagent N-ethylmaleimide was followed by incubation with NBD-Cl at pH 7.5. Binding of NBD to α and β of latent CF_1 is almost not diminished by this procedure (Table II, line 2).

The γ-subunit of latent, oxidized CF_1 contains two sulfhydryl groups of cysteines and one disulfide bond between two cysteines. On the latent enzyme, one of the two free sulfhydryls is accessible to the medium. The second is accessible only dur-

Table II. Quantitative evaluation of ^{14}C-labelled NBD bound to separated subunits of latent CF_1.

Conditions	mol [^{14}C]NBD/mol CF_1			
	Total CF_1	α_3	β_3	γ
Control	2.8 ± 0.8	0.9 ± 0.1	1.3 ± 0.7	0.6 ± 0.3
+ NEM	2.4 ± 1.0	1.1 ± 0.5	1.1 ± 0.6	0.3 ± 0.2
+ ADP + Mg^{2+}	1.5 ± 0.1	0.8 ± 0	0.4 ± 0.1	0.4 ± 0.2
Difference to control	1.3	0.1	0.9	0.2
+ ATP + Mg^{2+}	1.2 ± 0.2	0.2 ± 0.2	0.8 ± 0.1	0.3 ± 0.1
Difference to control	1.6	0.7	0.5	0.3

NBD (380 μM) incubation of isolated latent CF_1 (4 μM) at pH 7.5 was stopped after 1 h of incubation 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, the samples were reduced by 10 mM sodium dithionite for 10 min. ATP-, ADP-, and $MgCl_2$-concentrations were 5 mM, the final concentration of NEM was 10 mM. Other conditions see "Methods". The control and NEM-values were averaged from 4, the remaining values from 2 different experiments.

ing light activation of the ATPase (McCarty *et al.*, 1972). The NBD bound to γ may be bound to the accessible sulfhydryl residue, as the label in γ can be reduced to 0.1 NBD/CF_1 by alkylation with NEM. Binding of nucleotides, however, leads as well to diminuition of the label in γ. Activation of the enzyme (heat + DTT) previous to modification leads to three bound NBD molecules per CF_1 in one γ-subunit (Bickel-Sandkötter *et al.*, 1991). This additional NBD, which is bound after reduction of the disulfide bond in γ, cannot be found if the activated enzyme is exposed to NEM prior to NBD. The label in γ was reduced to almost zero under this condition (not shown). NEM does not inhibit the basic ATPase activity (own observation and Umbach *et al.*, 1990).

Furthermore, Table II shows, that Mg/ADP is able to decrease the label mainly in the β-subunits, whereas presence of Mg/ATP affects binding of NBD mainly to α. Diminution of the modifying NBD-label in β-subunits, obviously results in preservation of the activity (or preservation of the capability to become activated) of the enzyme. As can be deduced from Fig. 1 and Table II, only the NBD bound to β is related to the inactivation of the enzyme.

Determination of the NBD-labelled β-peptide by cyanogen bromide cleavement

[^{14}C]NBD-labelled β-subunits of latent, in the above described manner modified CF_1 (1 h incubation with NBD-Cl in presence or absence of ATP), were cut out of the gel (see "Methods") and cleaved with cyanogen bromide. As cyanogen bromide splits the polypeptide chain only on the carboxyl side of methionine residues (indicated by the scissors in the below diagram), one gets peptides of determined length. The resulting peptides were separated by SDS-polyacrylamide gels (see "Methods"). Fig. 2 shows the cleavage products of β-subunits, which were labelled with [^{14}C]NBD either in absence (lane 4) or in presence (lane 3) of ATP. The arrow indicates the peptide containing the radioactive label. For an example, the radioac-

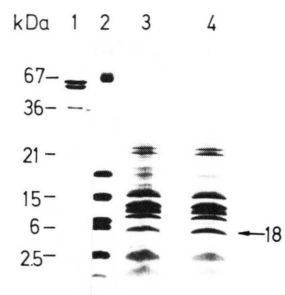

Fig. 2. Coomassie-blue stained PAGE of cyanogen bromide treated peptides of [^{14}C]NBD-labelled β-subunits (lane 3, NBD-incubated in presence of 5 mM ATP, lane 4, without ATP). The arrow indicates the peptide containing the radioactive label in either case. Lane 1, uncleaved CF_1, lane 2, protein molecular mass standard (Merck, Darmstadt).

Table III. Incorporation of ^{14}C into CF_1 and radioactivity of the different preparations.

Preparation	− ATP dpm/total preparation	− ATP nmol NBD/nmol protein	+ ATP dpm/total preparation	+ ATP nmol NBD/nmol protein
CF_1 after reduction and 1 × gel filtration	3023292.8	13.8 NBD/CF_1	2611012.8	14.0 NBD/CF_1
β-Subunits after cutting out of the gel, elution and lyophilization	123808.0	0.9 NBD/3β (NBD/CF_1)	127644.0	1.3 NBD/3β (NBD/CF_1)
β, after BrCN-cleavement, sum of all peptides out of the gel	16340.2	0.04 NBD/β	14219.7	0.06 NBD/β
Peptide β18	5531.8	0.031 NBD/β18	4468.3	0.038 NBD/β18

tivity of either preparation of the here shown experiment is shown in Table III. It may be seen from Table III, that most loss of radioactivity occurs during the step of BrCN-cleavement in 70% formic acid. But the remaining label in the β-subunits is clearly bound to peptide β18 in both cases, as shown in Fig. 3.

After being cut off and solubilized, the peptides were analyzed by liquid scintillation counting. Fig. 3 shows the radioactivity profiles of both cleaved β-preparations by plotting dpm against the peptide bands. In either case (incubated with ATP/not incubated) the radioactivity was found in peptide β18 (indicated by the arrow in Fig. 2). After blotting both bands of β18 to PVDF-membrane, the peptides were transferred to a gas phase sequencer for amino acid sequence analysis. The sequencing process was continued up to 23 cycles. The result showed that β18 was identical with a peptide containing the amino acids gly307 to met374. This peptide has a calculated molecular mass of 7 kDa and contains only two tyrosines, tyrβ328 and tyrβ362, as shown in the following diagram:

M ✂ GSLQERITSTKEGSITSIQAV **Y** VPADDLTDPAPATTFAHLDATTVLSRG–
307.....328.....
LAAKGI **Y** PAVDPLDSTSTM ✂
.....362.....374

Three separate analyses of different preparations of the labelled cyanogen bromide peptides yielded in exactly the same sequence. Contaminating peptides were not detected.

Table IV summarizes the sequencing results beginning with cycle 17: in the ATP incubated peptide tyr328 was modified. No phenylthiohydantoin derivative could be detected in cycle 22 (Ta-

Table IV. Amino acid sequence analysis of the labelled cyanogen bromide peptide β18. The results are shown starting with cycle 17.

β18 Cycle	**− ATP** Amino acid	Yield	β (323−331)	**β18** Cycle	**+ ATP** Amino acid	Yield
17	Ser	2.0	Ser	17	Ser	1.6
18	Ile	3.1	Ile	18	Ile	2.0
19	Gln	1.6	Gln	19	Gln	1.6
20	Ala	3.1	Ala	20	Ala	7.3
21	Val	3.1	Val	21	Val	3.2
22	**Tyr**	**2.7**	**Tyr**328	**22**	—	**0**
23	Val	3.4	Val	23	Val	2.7
			Pro	24	Pro	3.7
			Ala	25	Ala	4.1

The analysis was performed on about 100 pmol peptide. Lane 4 shows the amino acid sequence of a comparable peptide deduced from the nucleotide sequence of the chloroplast β-subunit (Walker *et al.*, 1985).

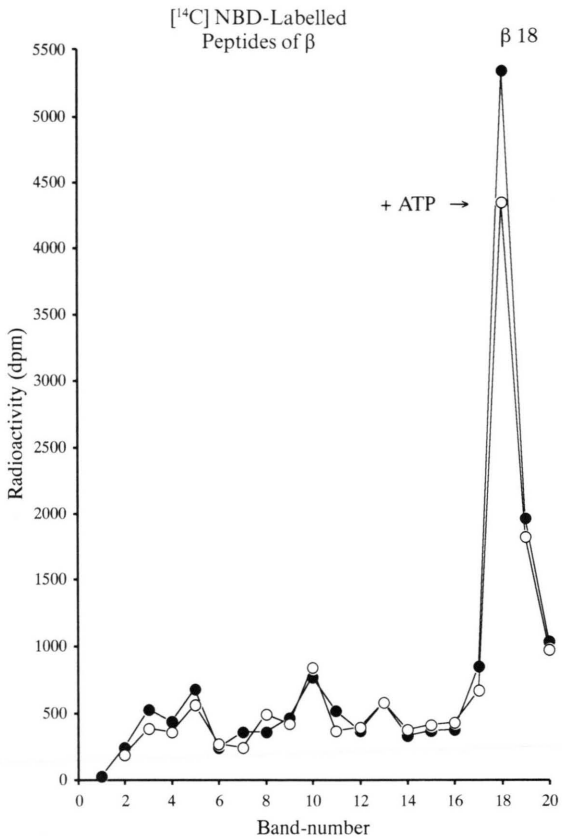

Fig. 3. Radioactivity profile of [^{14}C]NBD-labelled β-peptides of control and ATP-incubated CF_1. The peptide bands were cut out of the gel (20% polyacrylamide) dissolved in H_2O_2 as described in Materials and Methods and the radioactivity was measured by liquid-scintillation counting. The peptides corresponding to the indicated bands were blotted and transferred to a gas-phase sequencer.

ble IV). The sequencing process in this case was continued up to 25 cycles. This result confirms the already published finding of Ceccarelli *et al.* (1989), namely, that Tyr328 is modified with NBD.

The question concerning the stoichiometry of 1 NBD/3 β-subunits in context with the total loss of the amino acid in cycle 22 may be answered by the finding that different peptide pattern where found comparing modified and unmodified cyanogen bromide cleaved CF_1. Modification may alter the accessibility of residuals to the cleaving reagent.

In the control peptide, which was modified in **absence** of ATP, tyr328 (cycle 22 in Table IV) was found to be **unmodified,** as this tyrosine appeared in the normal amount. If it was modified, no, or at least a smaller amount of the phenylthiohydantoin derivative of the tyrosine would be detected in cycle 22. The result thus is consistent with the modification of tyr362, as the radioactivity was clearly found in this investigated peptide, and there are only two tyrosines in the analyzed peptide, as shown in the diagram. Under the employed conditions, lysines have not been modified (compare Bickel-Sandkötter and Strümper (1989a) and Bickel-Sandkötter *et al.* (1991)). Any N-lys-NBD-enzyme can be easily detected by the characteristic fluorescence of the sample and of the peptides within the gel (excitation maximum at 468 nm, emission maximum at 522 nm Ferguson *et al.*, 1975).

Digestion of labelled β-subunits with V8 protease

Though there is no doubt on the localization of the label in this peptide and there is only one tyrosine left which can bear the NBD, we ensure the deduced finding that tyr362 is the NBD-labelled amino acid in the following experiment. Labelling CF_1 by NBD-Cl as described above and digestion of the whole β-subunit with *Staphylococcus aureus*

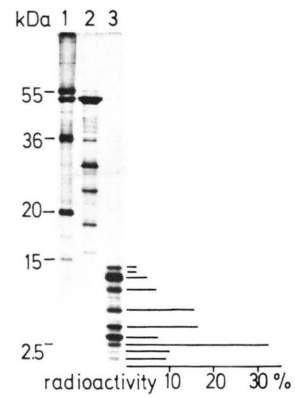

Fig. 4. Silver stained PAGE of *Staphylococcus aureus* protease (strain V8)-treated peptides of [^{14}C]NBD-labelled β-subunits (lane 3, 10 µg). Incubation with the protease was achieved in sodium phosphate buffer (20 mM) pH 8, containing 0.05% SDS, V8 protease was added to a final ratio (protease: CF_1) of 1:20; incubation at 37 °C, 20 h. Lane 1; 5 µg of uncleaved CF_1, lane 2: 5 µg of isolated β-subunits incubated 20 h in the digestion buffer (control). Right side diagram: Radioactivity profile of [^{14}C]NBD-labelled β-peptides (corresponding coomassie stained gel, % of total radioactivity of the layered sample, 100% = 2013 dpm).

protease (V8), resulted in a lot of small peptides, as shown in Fig. 4. V8 protease in phosphate buffer pH 8 cuts proteins behind the amino acids asparagine and glutamine. The digestion was almost complete after 22 h.

Following the above described procedure, the peptide containing the [^{14}C]NBD label was determined, the radioactivity profile of the peptides is shown by the bars on the right side.

The 2–3 kDa-peptide containing the most radioactive label was blotted to PVDF membrane and transferred to a gas phase sequencer for amino acid sequence analysis. The determined band of the gel, however, contained two peptides with a molecular mass (calculated) of 2.25 (1) and 2.2 kDa, (2) in the following diagram. One of the peptides (2) does not contain a tyrosine. The second one contains the above described tyr362 as the only one, which then must be the radioactively labelled amino acid. A parallel sample preincubated with 1 mM ATP showed no radioactivity in this peptide.

(1) D ✂ ATTVLSRGLAAKGI **Y** PAVD ✂ PLD
348.....362.....369
(2) E ✂ VIDTGAPLSVPVGGPTLGRIFNVLGE ✂ PVD
91.....116.....119

Table V shows the corresponding sequencing data of the first eight cycles.

Table V. Amino acid sequence analysis of the NBD-labelled, V8 protease digested β-peptide. Labelling with [^{14}C]NBD was carried out in the absence of ATP.

β10:1, 60%			**β10:2, 40%**		
Cycle	Amino acid	Yield [pmol]	Cycle	Amino acid	Yield [pmol]
1	Ala	59.9	1	Val	34.8
2	Thr	39.2	2	Ile	22.2
3	Thr	42.0	3	Asp	21.7
4	Val	36.1	4	Thr	32.5
5	Leu	20.5	5	Gly	16.3
6	Ser	8.5	6	Ala	13.5
7	(Arg)	–	7	Pro	13.0
8	Gly	15.5	8	(Leu)	0

The analysis was performed on about 120 pmol peptide. Peptide β10:1 consists of amino acids 348–366, β10:2 of amino acids 91–116.

Discussion

Our findings demonstrate that treatment of latent CF_1 with NBD-Cl inhibits the ATPase activity. The inhibition is accompanied by modification of two tyrosines, one per α- and one per β-subunit and one cysteine per γ-subunit. One NBD/CF_1, however, is sufficient for the complete inactivation of the ATPase activity (Bickel-Sandkötter and Strümper, 1989a; Bickel-Sandkötter *et al.*, 1991; Ceccarelli *et al.*, 1989). Complete inactivation of the enzyme is related to modification of one β-subunit out of three, though more NBD can be bound to CF_1, depending on the incubation time (Bickel-Sandkötter *et al.*, 1991).

Khananshvili and Gromet-Elhanan (1982) found modification of isolated β-subunits of *Rhodospirillum rubrum* chromatophores in a stoichiometric ratio of 1 NBD/β-subunit. One CF_1 contains three copies of β, therefore one could expect a stoichiometry of 3 mol NBD bound to 1 mol of CF_1, on β-subunits. The finding that latent CF_1 contained only 1 NBD/3β, when Ca-ATPase activity was completely inhibited, is in line with the idea that the ATPases contain three catalytic sites which are in three different states at the same time (Boyer and Kohlbrenner, 1981). If one of these sites becomes accessible to the modifying reagent, the whole catalytic cycle may become blocked.

Complete inactivation of bovine heart ATPase proceeding with the modification of one of the three β subunits was already reported by Ferguson *et al.* (1975), who concluded, that there must be an asymmetry within the enzyme concerning the three binding places on the three β subunits. The same was suspected by Stan-Lotter and Bragg (1986) who found that NBD and DCCD labelled different β-subunits of *E. coli* ATPase.

Andrews *et al.* (1984) concluded from their observations that βtyr311 in MF_1 (corresponds to βtyr328 in CF_1 and βtyr297 in EF_1) may not reside at the catalytic site, since modification of that residue by NBD affected ATP-hydrolysis more than

ATP-synthesis. We have tried to test the effect of NBD on photophosphorylation by addition of the reagent to chloroplast thylakoids. This procedere turned out to be not very useful, since NBD showed a strong effect on coupled and uncoupled electron transport. The isolated F_1 part of the chloroplast ATPase, however, is not able to synthezise ATP. But as ADP is a potent protector against modification and inactivation (Table I and II), whereas ATP is not able to protect the enzyme against NBD, it may well be that NBD also in CF_1 affects ATP hydrolysis more than ADP phosphorylation.

NBD-Cl modified the analogous tyrosines tyr311 in MF_1 (Andrews *et al.*, 1984) tyr307 in TF_1 (Verburg *et al.*, 1986) and tyr328 in chloroplast ATPase (Ceccarelli *et al.*, 1989 and this paper), inactivating either ATPase. Our results show that tyr328 is the modified amino acid residue if ATP/Mg is present in the modifying medium. This finding delivers the reason for the inability of the substrate ATP to protect the enzyme against inactivation. We conclude, again in accordance with Andrews *et al.*, that tyr328 cannot directly be involved in binding of ATP, at least in the latent state of the enzyme. But, despite of this, it must be essential for the activity (or for the activation) of the chloroplast ATPase.

Khananshvili and Gromet-Elhanan (1983) concluded from their reconstitution experiments with NBD-modified β-subunits and β-less chromatophores, that the site responsible for inactivation by NBD-Cl cannot be located on β, as the reconstituted particles showed restored activities of ATP-hydrolysis and -synthesis. This is controvers to our findings. Binding of NBD to α-subunits could be suppressed by previous incubation with ATP, but the enzyme remained inhibited. This is in accordance with the results of Andrews *et al.* (1984), who reported, that the incorporation of NBD into α of MF_1 did not correlate with the inactivation which they have observed. We conclude from our results, that the position of the MgATP-protectable tyrosine in α may belong to a structurally or regulatory nucleotide binding place of the ATPase. The remaining explanation for the discrepancy between the results of Khananshvili and Gromet-Elhanan and other groups may be found in different accessibilities of tyrosines in isolated β-subunits compared to intact F_1-parts of ATPases.

In both, membrane-bound (Bickel-Sandkötter and Gokus, 1989) and isolated (Horbach *et al.*, 1991) chloroplast ATPases resulted labelling with pyridoxal phosphate, which binds covalently to amino groups of lysines, in modification of one β-subunit (together with one α-subunit) in a completely inhibited enzyme. Moreover, the enzyme binds a maximum of 6 PLP/CF_1, but addition of substrates (ADP or ATP) prior to modification protected just one of the three sites of β yielding full activity of the enzyme. Both, hydrolysis (Bickel-Sandkötter *et al.*, 1991) and synthesis of ATP (Bickel-Sandkötter and Gokus, 1989) have been found to be affected equally by binding of PLP to the enzyme. In latent CF_1, βlys359 has been detected to be modified with PLP (Horbach *et al.*, 1991).

If no exchangeable nucleotides are bound to CF_1, tyr362 is labelled by NBD. This tyrosine, only two amino acids apart from lys359, is as well protected against modification by ATP. It is the one described by Xue *et al.* (1987a) to be labelled by the photolabelling nucleotide analogue 2-azido-ADP. The 2-azido-ADP had been derived from hydrolysis of 2-azido-ATP prior to photolabelling. The corresponding tyrosines in MF_1 (Wise *et al.*, 1987) and EF_1 (Cross *et al.*, 1987) have been reported to be also labelled by 2-azido-ATP. Tyr362 could also be covalently bound to benzoyl-ATP by Admon and Hammes (1987). Thus tyr362 may belong to a catalytic binding site of CF_1, though it cannot be excluded, that it occupies a position within β, which allows to influence the catalytic site inducing conformational changes. Our results suggest that binding of MgATP to the part of the enzyme containing tyr362, alters the conformation of the protein in a way that tyr328 (either in the same subunit, or in a second one) gets accessible.

Both tyrosines, tyr362 and tyr328 can be protected by preincubation of the enzyme with ADP. There are two possible interpretations: 1. Binding of ADP to the enzyme closes the catalytic cleft in a way, that no essential tyrosine in β can be reached by the modifying reagent. 2. ADP, in contrast to ATP, is bound to both places, the first containing tyr328 and the second containing tyr362, thus inhibiting the modifying reagent to get access to either tyrosine. The first interpretation corresponds to earlier findings of tightly bound ADP closing the catalytic binding site and hereby inacti-

vating the enzyme (Bickel-Sandkötter and Strotmann, 1981; Bickel-Sandkötter, 1983; Strotmann *et al.*, 1981). The second one may be consistent with the model published by Cross *et al.*, (1987) containing two nucleotide binding sites in one β-subunit. In this model, tyr385 (368 in MF_1) contributes to a non-catalytic ADP-binding site whereas tyr362 (345 in MF_1) in the same subunit is a part of the catalytic site, binding ADP or ATP. We could not find any NBD bound to βtyr385 on isolated, latent CF_1, using incubation times up to 90 min. This means that tyr385 in CF_1 is not easily accessible for the modifying reagent, which may be either due to chemical (surrounding medium) or to physical conditions.

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