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Identification of an essential β chain lysine residue from bovine heart mitochondrial ATPase specifically modified with nitrobenzofurazan

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A tetrapetide containing an essential lysine residue chemically modified with the nitrobenzofurazan group has been purified from bovine heart mitochondrial ATPase. The composition of the peptide indicates that this lysine is residue 401 in the sequence of a β chain. The modification was achieved by incubation at pH 9 of ATPase that had been previously labelled on a single essential tyrosine residue by reaction of the enzyme with 4-chloro-7-nitrobenzofurazan. The specific transfer of the nitrobenzofurazan group from the tyrosine residue to a particular lysine residue is consistent with the previously demonstrated intramolecular character of this transfer reaction.

Mitochondrial ATP synthase *Chemical modification* *Peptide purification* *Intramolecular shift* *F_oF_1-ATPase inhibition* *4-Chloro-7-nitrobenzofurazan*

1. INTRODUCTION

The mitochondrial ATP synthase enzyme can be separated into two structurally distinct parts, a water-soluble portion which has catalytic activity, usually termed F_1ATPase, and a membrane-bound sector which is a proton-translocating channel, usually called Fo. The F_1ATPase is generally accepted to comprise 5 types of polypeptide (α–ϵ) with stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [1–2]. F_1ATPase, as well as the complete ATP synthase, undergoes an unusual chemical modification reaction. At close to neutral pH the oxygen atom of a single tyrosine residue displaces chloride by nucleophilic attack from 4-chloro-7-nitrobenzofurazan (Nbf-Cl) to give Nbf-*O*-tyrosyl-F_1ATPase [3–5]. This modified enzyme is inactive [3–4]. An identical modification reaction has also been observed with F_1ATPases and ATP synthases from bacteria and thylakoids [6–9], suggesting that the reactivity of this tyrosine residue, and presumably its microenvironment, is highly conserved.

When mitochondrial Nbf-*O*-tyrosyl-F_1ATPase is incubated at pH 9.0 a substantial fraction of the Nbf moiety undergoes a transfer to the side chain of an amino acid that has been concluded to be lysine of a β chain on the basis of spectroscopic evidence [10]. The resulting Nbf-*N*-F_1ATPase is inactive [10]. The transfer can be followed from the disappearance of absorbance at 385 nm owing to Nbf-*O*-tyrosyl and the increase in absorbance at 475 nm, which is characteristic of amino-substituted Nbf [11]. Kinetic studies of the absorbance change at different concentrations of enzyme have shown the transfer to be intramolecular [10].

Further understanding of the possible role of the tyrosine and lysine residues to which the Nbf group can be attached requires identification of the modified residues within the amino acid sequence. This is not an easy task because of the lability of

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Abbreviations: Nbf-Cl, 4-chloro-7-nitrobenzofurazan; Nbf, nitrobenzofurazan

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the Nbf-*O*-tyrosyl species [4] and the large molecular mass, approx. 360 kDa, of the F_1ATPase. The former factor has caused other investigators to stabilise by chemical reduction the Nbf-*O*-tyrosyl group before fragmenting the enzyme [12,13]. Conflicting results have been obtained [12,13]. We have chosen to study first the location of the Nbf group after it has undergone intramolecular transfer. The amino-Nbf bond is relatively stable and therefore is easier to handle than Nbf-*O*-tyrosyl. Nevertheless, the method developed here might also be applicable to the difficult task of isolating a peptide containing the Nbf-*O*-tyrosyl species.

2. MATERIALS AND METHODS

Pepsin was purchased from Sigma, Nbf-Cl from Aldrich and [U-^{14}C]Nbf-Cl (109 Ci/mol) was from CEA (France) via Fluorochem Ltd, Dinting Vale, Glossop, England. Formic acid was redistilled from A.R. Grade. Trifluoroacetic acid was obtained from Pierce and acetonitrile, far UV grade, from Fisons. Sephadex G25 superfine was bought from Pharmacia and the Zorbex C8 column from Dupont.

All spectrophotometric measurements were made using a Kontron Uvikon 810 instrument. Bovine heart mitochondrial F_1ATPase was prepared as described in Sutton and Ferguson [5]. A molecular mass of 360 kDa for F_1ATPase was used in all calculations. Protein was assayed by a dye binding method using Coomassie blue [14]. Nbf was determined using extinction coefficients of 11600 $M^{-1} \cdot cm^{-1}$ [4] and 26000 $M^{-1} \cdot cm^{-1}$ [15] from Nbf-*O*-tyrosyl and amino-Nbf respectively. HPLC was carried out using Waters Associates pumps and model 660 solvent gradient programmer. The eluate was monitored using a variable wavelength UV detection system of Cecil Instruments. Amino acid analyses were made on hydrolysates (18 h treatment with 6 M HCl containing 0.5% phenol) of peptides using a Beckman 4400 automated analyser.

3. RESULTS

3.1. *Labelling of F_1ATPase on a specific lysine by Nbf*

100 nmol of F_1ATPase were desalted on a column of Sephadex G25 (fine grade) equilibrated with 0.25 M sucrose, 50 mM triethanolamine–HCl, 4 mM ATP, 2 mM EDTA adjusted to pH 7.5 with NaOH (buffer A) and diluted to 3.6 $mg \cdot ml^{-1}$ with buffer A. [^{14}C]Nbf-Cl (spec. act. 30 $Ci \cdot mol^{-1}$) was added to a final concentration of 100 μM and the solution incubated at 30°C in the dark. A 1 ml aliquot was transferred to the spectrophotometer and the progress of the reaction was followed at 385 nm. After 60 min the reaction was close to completion and the entire sample was transferred to a light-proof tube containing 4 g $(NH_4)_2SO_4$. The resulting suspension of protein was left to stand for 15 min at 0°C. This ammonium sulphate suspension was then centrifuged for 5 min in a microfuge at 4°C. The pellet was resuspended in a minimal volume of buffer A and desalted on a column of G25 Sephadex equilibrated in buffer A. At this stage the yield of F_1ATPase was 90 nmol and contained 1 mol of label bound to the tyrosyl residue and 0.1 mol bound to a lysyl residue per mol of enzyme as judged by the absorbances at 385 and 475 nm, respectively. Measurement of radioactivity indicated a total incorporation of 1.1 mol Nbf per mol of F_1ATPase.

Next, the pH of the solution of Nbf-modified ATPase (4.5 mg protein $\cdot ml^{-1}$) was raised to 9.0 by addition of triethanolamine. After 15 h incubation at 30°C the visible absorption spectrum [10] showed that 0.8 mol per mol of F_1ATPase of nitrobenzofurazan was bound to a lysyl residue and none to a tyrosyl residue. Analysis of the ^{14}C content of 5 nmol of the enzyme showed that 0.8 mol Nbf per mol of enzyme was bound.

3.2. *Digestion of Nbf-N-F_1ATPase with pepsin*

Formic acid was added to the remaining 85 nmol enzyme to a final concentration of 15% (v/v) and the sample dialysed against 800 vols 1% (v/v) formic acid at 4°C. At the end of the dialysis the precipitated enzyme contained 65 nmol nitrobenzofurazan, as judged by the ^{14}C content. 200 μg pepsin was added to the precipitated enzyme. After incubation for 5 h at 30°C the precipitate had disappeared reflecting the greater solubility in 1% (v/v) formic acid of the pepsin-generated peptides. The absorbance at 475 nm of this material indicated that it contained 65 nmol of amino-Nbf label.

3.3. *Gel filtration of pepsin-generated peptides*

The pepsin-digest was lyophilised, dissolved in 2 ml 1% (v/v) formic acid and chromatographed on a column of Sephadex G-25 (superfine grade) equilibrated in 1% (v/v) formic acid. The elution profile is shown in fig.1. Of the 50 nmol Nbf recovered from the column, 30 nmol eluted in fractions 85–95 (peak B). This elution position was later than that observed for cobalt acetate which indicates that material in these fractions was retarded by attractive forces between the column matrix and the Nbf group. Table 1 shows that 60% of the recovered Nbf group was in the peptides eluting in fractions 85–95.

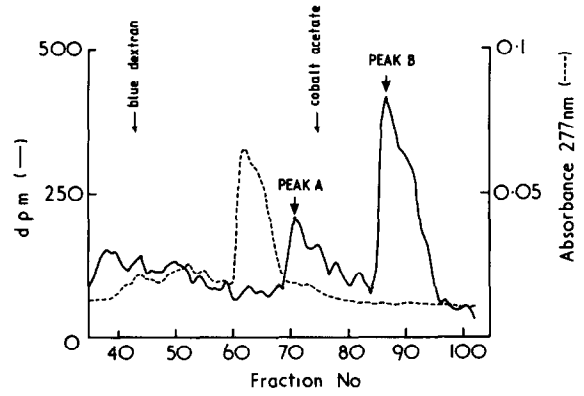

Fig.1. Chromatography of pepsin-generated peptides of Nbf-labelled F_1ATPase on Sephadex G-25. 65 nmol labelled F_1ATPase in 1% formic acid was loaded onto a column (160 × 2.2 cm) that contained Sephadex G-25 superfine equilibrated with 1% (v/v) formic acid. 5.8 ml fractions were collected and the absorbance was continuously monitored at 277 nm. 10 μl was taken from each fraction for determination of [^{14}C]Nbf content by scintillation counting. The elution positions of blue dextran and cobalt acetate were determined in a separate run.

3.4. *High-performance liquid chromatography (HPLC) of peak B fractions*

Fractions 85–95 from the Sephadex G-25 column were pooled, lyophilised, dissolved in 0.1% trifluoroacetic acid and chromatographed on a Zorbex C8 column. The elution profile is shown in fig.2. The amino-Nbf group was monitored at its shorter wavelength maximum of 345 nm [10] rather than at 475 nm. The profile of absorbance at 345 nm shows that the Nbf-containing material eluted as a single peak, indicative of a single peptide. The material collected in this peak was yellow/orange in colour with an absorbance maximum at 475 nm, which is characteristic of amino-Nbf [10], as was the colour of the fluorescence emitted from the sample. The absorbance at 280 nm relative to 345 or 475 nm was low whereas a spectrum of peak B from the Sephadex column showed that its absorbance at 280 nm was comparable with that at 475 and greater than at

Table 1

Purification of the Nbf-labelled peptide

Stage in preparation	nmol [^{14}C]Nbf	% yield	
		Of stage	Normalised relative to starting material
F_1ATPase labelled on tyrosine by Nbf	90	–	100
After transfer of Nbf to lysine	72	80	80
After digestion with pepsin	65	96[a]	76
Gel filtration			
fractions 85–95 (peak B)	30	77	35
fractions 70–77 (peak A)	15		18
fractions 35–55	5		6
1st HPLC	25	83	30
2nd HPLC	15	75[b]	23

[a] Calculated on basis that only 68 nmol of Nbf-*N*-F_1ATPase was digested with pepsin
[b] Calculated on basis that 20 nmol was subject to 2nd HPLC step

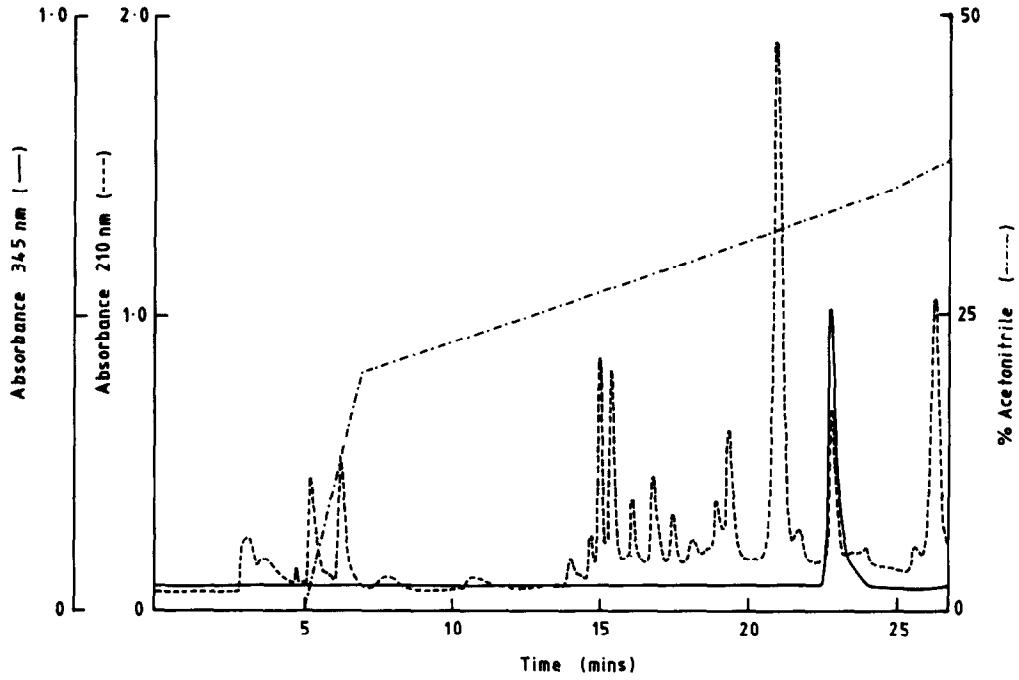

Fig.2. Elution profile of peak B fractions on HPLC. The absorbance at 345 nm (——) was determined in a separate run from that in which the absorbance at 210 nm (- - -) was followed. After loading the sample the column was washed for 5 min with 0.1% aqueous trifluoroacetic acid. Then gradients of acetonitrile in 0.1% trifluoroacetic acid (-·-·-) were applied; 0–20% (2 min); 20–40% (20 min). Application of a further gradient of acetonitrile, 40–90% (3 min) followed by 90% acetonitrile in 0.1% trifluoroacetic acid (3 min) and 90–0% acetonitrile in 0.1% trifluoroacetic acid (2 min) did not elute any additional material containing the Nbf group. Flow rate of the column was 1 ml·min^{-1}.

345 nm. These observations are consistent with purification by HPLC of a peptide that lacked aromatic amino acids (table 2). Amino acid analyses of fractions collected within this peak are shown in table 2. The lysine content was low relative to threonine, valine and leucine. This was attributed to only partial degradation of a nitrobenzofurazan-lysine bond during hydrolysis of the peptide. The amino acid analysis revealed an unknown species which was presumably Nbf-*N*-ϵ-lysine. Table 2 shows that the relative content of glycine was high and thus the peptide was again subjected to HPLC, but using a shallower gradient of 20–35% acetonitrile with a run time of 30 min rather than the conditions shown in fig.2. An amino acid analysis of the repurified peptide is shown in table 2. The glycine content, was reduced from the first analysis whilst the content of threonine, leucine, valine and lysine was very

Table 2

Amino acid analyses of purified Nbf-labelled peptide

Amino acid	After 1st HPLC		After 2nd HPLC	
	mol per mol Nbf	Residue per mol	mol per mol Nbf	Residue per mol
Thr	0.88	0.92	0.83	0.91
Ser	0.22	–	–	–
Glx	0.27	–	–	–
Gly	0.36	–	0.17	–
Val	0.97	1.01	0.95	1.04
Leu	1.01	1.06	0.95	1.04
Lys	0.53	0.56	0.62	0.64
Others	<0.2	–	<0.15	–

The Nbf content (2.8 nmol of sample from 1st HPLC and 3.57 nmol from 2nd HPLC) was calculated from the absorbance at 475 nm. Residue per mol is calculated relative to the three major amino acids

similar. It was concluded that the peptide contained the amino acids threonine, leucine, valine and lysine in equimolar quantities.

4. DISCUSSION

The data presented in table 2 show that a single tetrapeptide containing the Nbf group has been purified. Apart from lysine the other three amino acids present do not possess side chains that could be derivatised by Nbf and account for the absorbance maximum at 475 nm. This consideration, together with the substoichiometric amount of lysine determined in the amino acid analysis, attributed to partial breakdown of the Nbf-*N*-lysine species during the hydrolysis of the tetrapeptide, means that Nbf must have transferred to lysine upon incubation of Nbf-*O*-tyrosyl-F_1ATPase at pH 9.0. Thus the original conclusion [10], which was based on spectroscopic data alone, is justified.

Inspection of the amino acid sequence of the β chain of F_1ATPase [16] reveals that only lysine 401 could be part of a peptide containing equimolar valine, threonine and leucine. The sequence of the peptide is therefore lys-leu-thr-val. In agreement with this conclusion N-terminal analysis by dansylation of the tetrapeptide followed by acid hydrolysis gave no evidence for dansyl derivatives of leucine, valine or threonine.

The identified tetrapeptide contained approx. 60% of the [^{14}C]Nbf that was applied to the Sephadex G-25 column (fig.1). The composition of the material containing the remaining 40% has not been investigated but it could arise for three reasons: (1) incomplete pepsin digests; (2) incorporation of Nbf on lysine during the initial labelling of the F_1ATPase by Nbf-Cl; (3) transfer of Nbf group from Nbf-*O*-tyrosyl ATPase to lysine residues other than 401. However, the finding that at least 60% of the Nbf was found on lysine 401 substantiates the previous conclusion that an intramolecular transfer occurred. An intermolecular transfer would have resulted in a widespread distribution of Nbf amongst the lysine residues of F_1ATPase.

Lysine 401 is conserved in the enzyme from *Escherichia coli* but not by thylakoid F_1ATPase where it is replaced by arginine [16]. This might suggest that this residue is not catalytically essential, although for the purposes of binding a negatively charged substrate these two amino acids might be equivalent. However, lysine 401 lies in the middle of a highly conserved sequence in the F_1ATPases [16]. This indicates that this region of the polypeptide may have functional importance. From the comparative sequence data it might be predicted that the transfer of Nbf from tyrosyl to lysine would be similar to mitochondrial F_1ATPase for the *E. coli* enzyme but different or absent for the thylakoid enzyme. Information is limited on this point although the transfer for the *E. coli* enzyme has been reported to be slower than for the mitochondrial enzyme [8]. A transfer reaction has been reported for the thylakoid enzyme [17], but in common with the *E. coli* enzyme it has not been reported whether this is an intramolecular process.

Presumably the tyrosine residue to which Nbf initially attaches is within fairly close proximity to, and has good accessibility to, lysine 401. However, as shown elsewhere [5] lysine 401 must be sufficiently distant from the tyrosine to account for the absence of steric hindrance to reaction between the tyrosine of Nbf-*N*-F_1ATPase and Nbf-Cl. The location of the tyrosine within the β chain is uncertain because whereas Ho and Wang [12] have assigned it to tyrosine 197, Andrews et al. [13] have concluded that tyrosine 311 is modified. The strategy for identifying Nbf-containing peptides used here, and earlier in part by Aboderin and Boedefeld [18], uses acidic conditions in which the *O*-Nbf group is relatively stable. Hence extension of the approach used here may identify the reactive tyrosine without the need for reduction of the Nbf group [12,13]. It is possible that the reduction process is responsible for the two different locations reported for this tyrosine residue [12,13].

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