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Nucleotide binding to active and 4-chloro-7-nitrobenzofurazan-inhibited forms of chloroplast F_1-ATPase – an NMR study

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(1) Chloroplast F_1-ATPase (CF_1) was labelled on both a single tyrosine and at least two cysteine residues by 4-chloro-7-nitrobenzofurazan (Nbf-Cl). The ATPase activity was inhibited by 80–90%. Prior treatment of latent CF_1 with *N*-ethylmaleimide (NEM), to block accessible thiol groups, reduced the Nbf-cysteine modification to the equivalent of one residue per enzyme molecule. Only one tyrosine residue, identified here as tyrosine 328 on one beta subunit, is modified by Nbf-Cl. This is analogous to the tyrosine residue which reacts with Nbf-Cl on the related enzyme from mitochondria (MF_1). (2) ^{31}P and ^1H nuclear magnetic resonance (NMR) studies of CF_1 identified a single tight binding site for the Ca^{2+} complex of the ATP analogue 5′-adenylylimidodiphosphate (AMPPNP) on each of latent, trypsin activated, and NEM modified CF_1. No NMR signals could be detected from from this site. Tight binding of Ca-AMPPNP to latent and trypsin activated CF_1 was also inferred from its low inhibition constant ($K_i \approx 12$ μM). NMR also showed that addition of excess Ca-AMPPNP to both latent and NEM-labelled CF_1 displaced one tightly bound adenine nucleotide. The ^{31}P signals from nucleotides ADP or ATP bound at this single tight site were too broad to be observed but could readily be detected after denaturation of the enzyme with detergent. (3) In contrast to the active forms of CF_1, the Nbf-Cl modified enzyme was found to have one ADP and one ATP bound. ^{31}P-NMR signals from the ADP, but not the ATP, could be detected. These signals had the linewidths expected for a bound ligand. In the presence of excess Ca-AMPPNP the bound nucleotide slowly dissociated from the Nbf labelled enzyme at a rate which correlated with loss of the Nbf group from tyrosine 328. This was accompanied by a loss of signal intensity equivalent to one mol of Ca-AMPPNP per mol enzyme.

Introduction

The F_0F_1-ATP synthases of mitochondria, bacteria and thylakoids are closely related complex enzymes (for reviews, see Refs. 1 and 2). We are, however, still some way from understanding their molecular mechanisms. Among aspects not fully understood are (a) the role(s) of tightly bound adenine nucleoties; (b) the number of functioning active sites; (c) the reason for the asymmetric structure of the catalytic domain (F_1). The asymmetry in the catalytic portion of these enzymes is illustrated by the finding that incorporation of just one nitrobenzofurazan (Nbf) group per molecule of the mitochondrial F_1-ATPase (MF_1), on one of three beta chains, results in substantial (more than 99%) loss of activity [3–5]. The specific residue labelled by Nbf-Cl has been identified as tyrosine 311 in the mitochondrial enzyme, but the basis for the inhibition is unclear [6–8]. Site-specific mutagenesis of the equivalent tyrosine to phenylalanine does not significantly alter the activity of the *Escherichia coli* enzyme [9].

The present paper is concerned with the application of both ^{31}P- and ^1H-NMR to studies of ligand binding

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Abbreviations: CF_1, chloroplast F_1-ATPase; MF_1, mitochondrial F_1-ATPase; RUBISCO, ribulose-1,5-bisphosphate carboxylase-oxygenase; NEM, *N*-ethylmaleimide; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; Nbf, 4-nitrobenzofurazan; AMPPNP, 5′-adenylylimidodiphosphate; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; 2PDS, 2,2′-dithiopyridine; TFA, trifluoroacetic acid.

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to F_1-ATPase. The intention was to probe adenine nucleotide binding sites and to compare the binding properties of active enzyme with that modified by Nbf-Cl. The chloroplast enzyme was chosen for these studies because NMR experiments require large amounts of protein on a regular basis, which is a requirement most easily met from this source. The modification of CF_1 with Nbf-Cl was investigated because this reaction had not been as thoroughly studied for CF_1 as for MF_1. In contrast to MF_1, reaction of Nbf-Cl with thiol groups occurs but this can be reduced by blocking them by reaction with *N*-ethylmaleimide (NEM).

Materials and Methods

The following chemicals and enzymes were purchased from Sigma: Sephadex gel filtration and ion exchange gels, ADP, ATP, 5′-adenylylimidodiphosphate (AMP-PNP), EDTA, Tris, Tricine, NEM, Biuret reagent, Folin and Ciocalteau's phenol reagent, deuterium oxide, TPCK-treated trypsin, soybean trypsin inhibitor, 2,2′-dithiopyridine (2PDS), sodium dodecyl sulphate (SDS), bovine serum albumin, spinach chloroplast ribulose-1,5-bisphosphate carboxylase-oxygenase (RUBICSO) and pepsin.

The ATP used in assays was obtained from Boehringer Mannheim Biochemicals; Nbf-Cl from the Aldrich Chemical Company Ltd. and desferrioxamine from Ciba-Geigy Ltd. The C8-MOS Hypersil HPLC resin was supplied by Shandon Southern products and the C18 Partisil-10 ODS-3 resin by Whatman. All other reagents used were of AR grade, or, in the case of solvents, of HPLC or spectroscopic standard.

Latent CF_1 was isolated from fresh spinach leaves as described by Lien and Racker [10]. Activation of latent CF_1 by mild trypsin treatment was carried out essentially as described by Moroney and McCarty [11].

Samples of CF_1 from which all free nucleotides had been removed were prepared for NMR experiments by passing CF_1 solutions through a Sephadex G-25–150 gel filtration column, which was pre-equilibrated and eluted using a 40 mM Tricine (pH 7.6) buffer. Typically, about 2.5 ml of a 60 mg per ml protein sample was passed down a 15 ml column to ensure complete separation of free nucleotides from CF_1. The enzyme solution was then immediately frozen in liquid nitrogen and stored at −70 °C before NMR measurements.

The protein concentration of enzyme samples was determined using a modification of the Lowry procedure proposed by Ohnishi and Barr [12].

The calcium-ion-dependent ATPase activity of CF_1 samples was determined spectrophotometrically from phosphate release [13]. The assays were carried out at 37 °C in a pH 8.0 buffer containing 40 mM Tricine, 10 mM ATP and 10 mM $CaCl_2$.

The K_i for Ca-AMPPNP inhibition of latent or trypsin activated CF_1 was determined by measuring, at initial substrate concentrations between 1 and 7 mM, the effect of up to 160 μM inhibitor on the ATPase activity. The CF_1 samples, in a 40 mM Tricine and 10 mM $CaCl_2$ (pH 8.0) buffer, were initially incubated with AMPPNP for 1 h at 37 °C. The required amount of ATP was then added and the ATPase activity assayed as described above. The K_i values for Ca-AMP-PNP inhibition of CF_1 were determined by means of 'direct linear' plots of the data [14].

The reaction of latent CF_1 with NEM was carried out in a 40 mM Tricine and 1 mM ATP (pH 8.0) buffer using a 20-fold molar excess of NEM over protein for 2 h at room temperature. The enzyme was then separated from excess reagent on a Sephadex G-25–150 column.

CF_1 was labelled by Nbf-Cl in 40 mM tricine, 1 mM ATP (pH 8.0), with Nbf-Cl in a fivefold molar excess over protein. The progress of the modification reaction at room temperature was monitored by the increase in absorbance at 400 nm. On completion of the reaction the CF_1 was separated from excess reagent on a Sephadex G-25–150 column.

The assays for thiol groups were carried out using 2,2′-dithiopyridine, essentially as described by Grassetti and Murray [15].

Since RUBISCO was found to contaminate the CF_1 preparation, modification experiments were also carried out on a commercial preparation of this enzyme. The modification of RUBISCO with Nbf-Cl was carried out as described for CF_1. When dealing with RUBISCO samples that had not been pretreated with NEM, a 20-fold molar excess of reagent over protein was used.

The sites of Nbf-Cl labelling on NEM/Nbf-CF_1 were identified using a procedure based on that reported by Sutton and Ferguson [7,16]. The modified protein was digested in the dark for 4 h at 30 °C with pepsin in 1% formic acid (NEM/Nbf-CF_1 : pepsin = 82 w/w); separated on a 150 cm by 2.2 cm Sephadex G-25–50 gel filtration column eluting with 1% formic acid, and the elution profiles followed by measuring the absorbances at 280 and 385 nm. Low-molecular-weight fractions were pooled, freeze dried and dissolved in 1 ml of 0.1% trifluoroacetic acid (TFA). The pepsin digest was purified by HPLC using a C8 MOS Hypersil hydrophobic column equilibrated with a 98 : 2 mixture of buffer A (0.1% TFA in 95 : 5, water/acetonitrile): buffer B (0.1% TFA in 20 : 80, water/acetonitrile) and eluting with a linear gradient increasing buffer B from 2% to 80%. Small aliquots were separated and peaks containing peptides with modified cysteines and tyrosines were identified by comparing the peak intensities at 385 and 425 nm. The remainder of the digest was then passed through the C8 column and the Nbf-peptide fractions collected at 385 nm. The major Nbf-labelled tyrosine and cysteine peptide fractions were further purified on a

C18 Whatman Partisil-10 ODS-3 column using a linear gradient, increasing buffer B from 15% to 75%. Absorbance spectra were measured to confirm and quantify the type and amount of Nbf-peptide isolated. Amino-acid compositions were determined using the Waters Associates 'pico tag' procedure and were sequenced on an Applied Biosystems 470A gas phase protein sequencer.

The ^{31}P-NMR studies of CF_1 were carried out at 145.7 MHz. All measurements were carried out with 3 ml samples in 10 mm diameter NMR tubes containing an external chemical shift reference of methylene diphosphonic acid at pH 7.4 (17.13 ppm downfield of orthophosphoric acid). Sweep widths were 8000 Hz collected in 8 K data points. Typical CF_1 samples consisted of 80–110 mg per ml enzyme in a deuterated 40 mM Tricine buffer (pH 7.6).

The ^{1}H-NMR investigations of CF_1 were performed at 500 MHz using 5 mm samples tubes. Typical samples were 25 mg per ml enzyme, 20 mM potassium phosphate buffer. The spectra were recorded using a $90°$-τ-$180°$-τ spin echo pulse sequence with $\tau = 5$ ms.

Results

Characterisation of Nbf-Cl labelling of CF_1

Under aqueous conditions it is known that the reaction of the hydroxyl group of tyrosine with Nbf-Cl produces a derivative with a visible absorption maximum at 385 nm, whereas modification of the sulphydryl group of cysteine leads to a product with an absorption maximum at 425 nm [3–5,17,18]. Since in the present work (and cf. Deters et al. [19]) the reaction between Nbf-Cl and CF_1 gave a product with a maximum absorption at 410 nm, labelling of both tyrosine and cysteine residues was thought to occur. Pretreatment of CF_1 with NEM, to modify reactive thiol groups, caused no loss in activity, but resulted in the absorption maximum of the Nbf-derivatised enzyme shifting to 405 nm. This indicated that the reaction of cysteine with Nbf-Cl, although reduced, was still significant. An estimate of the relative amount of Nbf labelling of CF_1 on the two types of residue was obtained by using known molar extinction coefficients for each derivative at 385 and 425 nm and then solving simultaneous equations. Thus, the degree of Nbf labelling per latent CF_1 molecule was calculated to be 0.64 ± 0.06 on tyrosine and 2.0 ± 0.22 on cysteine. Equivalent values obtained for NEM-pretreated enzyme were 0.85 ± 0.09 and 1.02 ± 0.28, respectively. These estimates assume that all the protein present was CF_1. However, as explained later, there is evidence for contamination by RUBISCO. Under the conditions used here, 80–90% of the activity of both enzyme forms was lost after incubation with Nbf-Cl, as reported for both latent and trypsin activated CF_1 [19].

A peptide bearing the Nbf-derivatised tyrosine residue from MF1 can be purified by digesting the modified enzyme with pepsin and then purifying the resulting peptides by gel filtration and HPLC [7,16]. A similar procedure was applied here to NEM/Nbf modified CF_1, as outlined in the Materials and Methods section. After gel filtration and HPLC on a C8 column a number of fractions containing Nbf-labelled material could be identified. The amount of labelling was determined by weighing cut-out HPLC traces of absorbances at 385 nm, for Nbf-tyrosine, and 425 nm, for Nbf-cysteine. This provided an estimate for the ratio of total Nbf modified tyrosine to cysteine of 0.7, which is comparable with the observed labelling pattern on the intact enzyme.

The main Nbf-tyrosine fraction, identified from its absorbance at 385 nm and containing between 55% and 80% (depending on the experiment) of the total tyrosine-O-Nbf chromophore, was further purified on a C18 column (Fig. 1). In this way four fractions containing peptides were isolated but only one had a characteristic Nbf-tyrosine absorbance maximum at 375 nm in acetonitrile. Subsequent sequencing of this fraction showed that there were two peptides present. One of these contained no tyrosine residue and corresponded to residues 67–77 from the large subunit of RUBISCO [20]. The sequence of the second peptide was identical to that of residues 327 to 335 in the beta chain of CF_1 [21]. This sequence contains only one tyrosine residue at position 328, which therefore must be the site of Nbf-Cl modification.

The main Nbf-cysteine peptide isolated was also sequenced. It was identical to residues 243–249 from the large subunit of RUBISCO, with a cysteine at position 247 [20]. The failure to identify peptides from CF_1 containing Nbf-modified cysteine could be due to

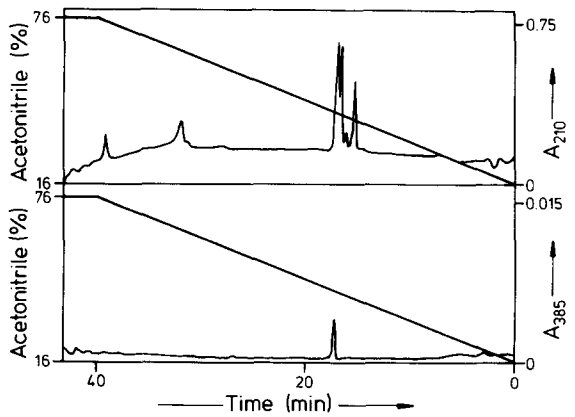

Fig. 1. An illustration of the reverse-phase HPLC separation, on a C18 column, of the main Nbf-tyrosine labelled peptide obtained from a pepsin digest of NEM/Nbf modified CF_1. The trace at 210 nm identifies all the peptide material present, whilst the one at 385 nm arises from peptides that cotnain an Nbf group, in this case Nbf-tyrosine.

the labelling being distributed over several cysteines, or pepsin digestion not giving defined labelled fragments.

These analyses show that a major locus of Nbf-Cl modification of CF_1 is a specific tyrosine residue. However, it is also apparent that the CF_1 is contaminated by RUBISCO. As reported previously [22,23] it is only possible to remove the RUBISCO from the CF_1 preparation by using an antibody-based affinity chromatography column. This procedure would be very difficult to apply for the large amounts of protein needed for the NMR studies. The extent of the RUBISCO contamination is not easy to gauge, but an estimate of 10% was suggested by a comparison of the staining intensities of polypeptides after separation by SDS-PAGE. Assays of the total number of thiol groups in pure RUBISCO and CF_1 preparation also indicated a contamination of around 10%. (Under non-reducing conditions the CF_1 preparation was found to have 17.5 $\pm$ 0.9 cysteines per enzyme molecule, compared to an expected 13 [21,24–26]. Pure RUBISCO was found to have a total of 50.5 $\pm$ 1.5 cysteines per enzyme molecule. The reaction of Nbf-Cl with pure RUBISCO labelled 5.2 $\pm$ 0.6 cysteines per enzyme molecule. This was reduced to 1.58 $\pm$ 0.3 by pretreatment with NEM. No tyrosine modification could be detected. From these three sets of data it is estimated that all of the tyrosine and most of the cysteine labelling (more than 75%) by Nbf-Cl occurs on CF_1, rather than on contaminating RUBISCO.)

AMPPNP binding and NMR properties of bound nucleotides

The calcium ion complex of AMPPNP, a non-hydrolysable ATP analogue, was shown to be a competitive inhibitor of both the latent and trypsin activated CF_1, with a K_i of about 12 μM. This is comparable to previously reported values [27].

A single tight binding site for metal ion complexes of ADP, ATP or AMPPNP has been previously identified on latent and dithiothreitol activated CF_1 by ^{1}H-NMR experiments [28,29]. Our ^{1}H-spin echo experiments gave very similar results; the ribose ring proton resonances of Ca-AMPPNP were only observed after more than 1 mol ligand per mol enzyme was present in the sample. The absence of signal from bound nucleotide is expected in this kind of spin echo experiment because of the short relaxation time [30].

Similarly, when increasing amounts of Ca-AMPPNP were added to solutions of either latent, NEM-modified or trypsin-activated CF_1, no ^{31}P resonances from Ca-AMPPNP were detectable until the concentration of this nucleotide exceeded that of CF_1 (Fig. 2). Subsequent additions were associated with linear increases in signal intensities which were very similar to those observed when Ca-AMPPNP was added to buffer alone (e.g., for latent enzyme in Fig. 2). A resonance assigned

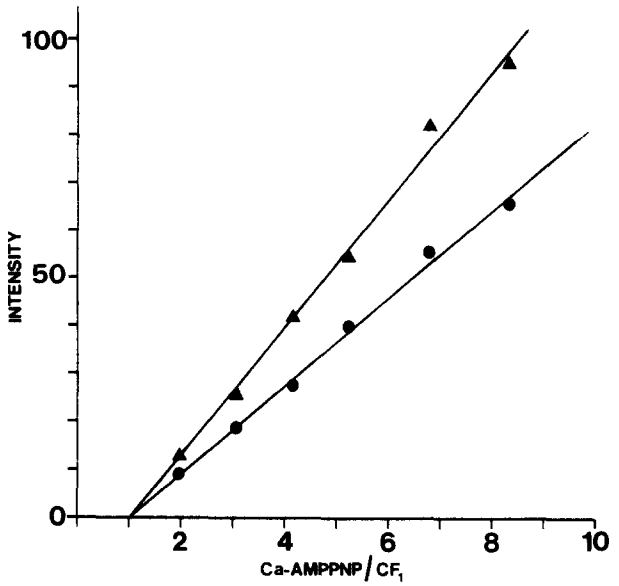

Fig. 2. Plots of the intensity (arbitrary units) of the ^{31}P-NMR signals from the beta (●) and gamma (▲) phosphate groups of Ca-AMPPNP as a function of the ratio of added Ca-AMPPNP to latent CF_1. The curves are a least-squares fit of the data to straight lines. No signals were detected until more than 1 mol Ca-AMPPNP per mol latent CF_1 was present. The sample contained 80 mg per ml latent CF_1 in D_2O/tricine buffer (pH 7.6); the pH was adjusted and the sample allowed to equilibrate for 1 h between each addition. The Ca-AMPPNP was added as equal aliquots from stock solutions of 0.1 M AMPPNP and 0.5 M $CaCl_2$. The spectra arose from 1000 transients.

to free AMP was observed to appear as Ca-AMPPNP was added, presumably arising from 'NMR invisible' bound nucleotide (see later). The total amount released corresponded to approx. 1 mol per mol enzyme and the release was complete after the addition of 1 mol Ca-AMPPNP.

^{31}P-signals from ligands bound to large proteins have been observed before although sometimes they have very long T_1-values [31]. The failure to detect them from CF_1 was thus surprising. Considerable effort was made in this study to detect signals from bound nucleotides. Long spectral accumulations under different conditions including small flip angles and long relaxation delays were tried without success. The basis for the failure to observe ^{31}P-NMR signals from Ca-AMPPNP bound to CF_1 was therefore investigated further.

The NMR experiments were performed with enzyme solutions of between 50 and 300 μM (20–120 mg per ml). Under the same conditions but without protein, Ca-AMPPNP could be detected at a concentration of 50 μM, after only 4000 transients, whereas 300 μM could not be observed after 150000 transients when bound. (Note that it is possible to observe the ^{31}P-NMR signals from a ADP molecule bound to NEM/Nbf modified CF_1, after only 4000 scans – see below.) The most likely explanation for the 'invisible' signal is that there is a single tight site on the enzyme where the ^{31}P-NMR signals become too broad to detect.

CF_1 might have had a paramagnetic ion, such as Fe^{3+} or Mn^{2+}, which could broaden the resonances at the Ca-AMPPNP binding site. Indeed it is known that CF_1 contains a potential tight manganese binding site (see, for example, Ref. 32). Addition of EDTA or desferrioxamine had no observed effect on the ^{31}P-spectra and no bound manganese was found previously with a similar preparation [32].

The ^{31}P-NMR signals might also be broadened by exchange between the bound and free forms of the nucleotide. At its maximum, this kind of exchange process can produce line-broadening that approaches the frequency difference between equivalent resonances in the two states. The chemical shifts and linewidths of the ^{31}P-resonances from free Ca-AMMPPNP, in an 85 mg per ml latent CF_1 sample (with Ca-AMPPNP/CF_1 molar ratios in the range 2:1 to 6:1), were indistinguishable from those observed for the same concentration of the ATP analogue in an 85 mg per ml BSA solution. The ^{31}P-NMR signals had a chemical shift within 0.05 ppm of that predicted by pH titration curves for Ca-AMPPNP [33]. Thus, in samples of latent, NEM-modified and trypsin-activated CF_1 there is no evidence to suggest that ^{31}P resonances from the initial molar equivalent of Ca-AMPPNP added are lost by exchange broadening.

Another possible exchange process is between the two enzyme-bound conformational states of Ca-AMP-PNP with different chemcial shifts. The ^{31}P-NMR results were, however, the same at both 20 and 37 °C. One might have expected, at the higher temperature, a faster exchange rate between such interconverting forms to give rise to sharper ^{31}P-NMR signals.

If aggregation of CF_1 were occurring under the conditions used in the ^{31}P-NMR studies then the resulting increase in linewidth for bound Ca-AMPPNP resonances might account for the inability to detect these signals. However, sedimentation velocity studies of latent CF_1 did not detect significant aggregation of the protein at concentration less than 40 mg per ml [33]. No ^{31}P-NMR signals from bound ligand were detected with enzyme solutions down to a concentration of 20 mg per ml.

To summarise, the failure to detect ^{31}P-NMR signals from Ca-AMPPNP bound to CF_1 is unlikely to be due to paramagnetic ions at the nucleotide binding site. Protein aggregation and exchange broadening between enzyme bound forms are possibilities, but the most likely explanation is that the chemical shift anisotropy contribution to the ^{31}P-linewidths is larger than expected, as outlined further in the discussion.

Bound nucleotides and the kinetics of their release

Although bound adenine nucleotides on latent and NEM-modified CF_1 could not be detected by ^{31}P-NMR even in spectra representing the sum of 120000 transients, denaturation of CF_1 by SDS resulted in the appearance of relatively sharp resonances (Fig. 3), which could be quantitated by adding known amounts of ADP or ATP. Experiments of this type reproducibly showed that latent CF_1 initially contained about one ATP per enzyme molecule. While ATP could be detected by performing the denaturation experiment quickly, the ATP was hydrolysed to ADP and inorganic phosphate, as reported previously [34], if the native enzyme samples were left at room temperature for several hours. Latent CF_1 that had been treated with NEM was also found to have one bound nucleotide under the conditions used.

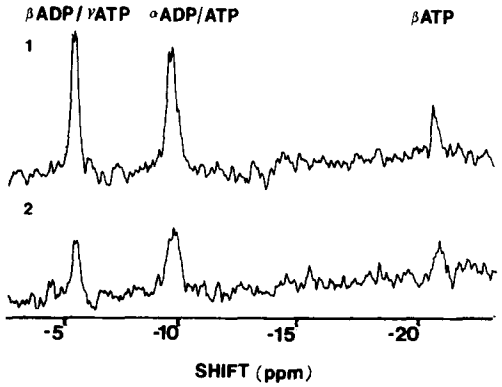

Fig. 3. ^{31}P-NMR spectra acquired following denaturation of (1) NEM/Nbf-modified CF_1 and (2) latent CF_1, by the addition of SDS to a final value of 0.5% (w/v). Further addition of SDS up to 2% made no difference to the spectrum. The enzyme concentration was approx. 60 mg protein per ml. Free nucleotides were removed by gel filtration before denaturation. The spectra have been normalised to account for a slight difference in the enzyme concentration. Note the additional bound ADP molecule seen in the NEM/Nbf-labelled CF_1.

In ^{31}P-NMR studies with both latent and NEM-modified CF_1 no dissociation of nucleotides or inorganic phosphate from the enzyme could be detected even after 48 h. This confirms that the ligands were tightly bound to the enzyme. However, when a threefold molar excess of Ca-AMPPNP was added then all the bound adenine nucleotide was released, as AMP and inorganic phosphate, within the time required to obtain a ^{31}P-NMR spectrum (Fig. 4). This is consistent with an exchange of free Ca-AMPPNP for nucleotide bound on CF_1, as reported previously [34–36]. The appearance of AMP is attributed to the action of contaminating adenylate kinase coupled to the ATPase activity of CF_1 [37].

The release of AMP and inorganic phosphate, upon addition of Ca-AMPPNP, was followed by a slow appearance of additional inorganic phosphate. This is almost certainly due to a slow hydrolysis of Ca-AMP-PNP catalysed by CF_1, comparable to the action of MF_1 on this ATP analogue [38].

While latent and NEM-modified CF_1 each contained a single tightly bound adenine nucleotide from which

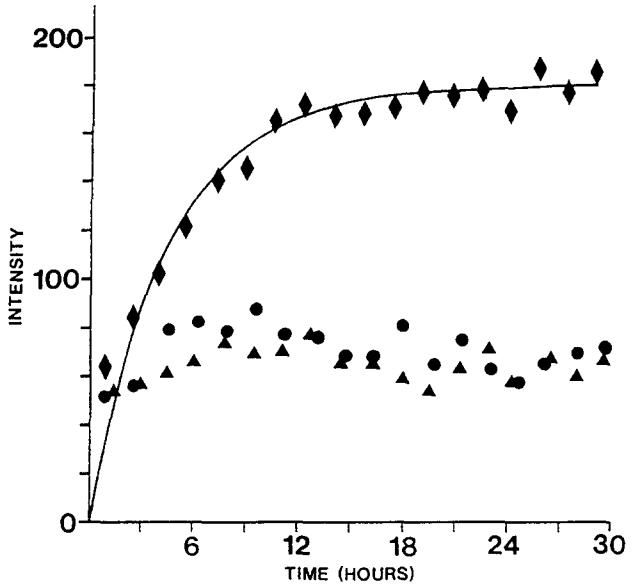

Fig. 4. Plots of the intensity of the ^{31}P-NMR resonance from free AMP as a function of time, in solutions of latent (▲), NEM treated (●) and NEM/Nbf-modified CF_1 (◆) (Ca-AMPPNP/CF_1 ratio of 3:1 (pH 7.6), 20 °C). Peak intensities were normalised to account for differences in enzyme concentration. The curve through the points for NEM/Nbf-labelled CF_1 is the best fit of the data to a first-order process. Note that about twice as much nucleotide is released from the NEM/Nbf-modified enzyme. In the latent and NEM-modified enzymes the release appears to occur relatively rapidly.

^{31}P-resonances could not be detected, ^{31}P-NMR signals arising from the alpha- and beta-phosphate groups of a bound ADP molecule on NEM/Nbf modified CF_1 could be seen in spectra consisting of only 4000 transients (Fig. 5). The widths of these resonances were around 160 Hz for the alpha-phosphorus and 125 Hz for the

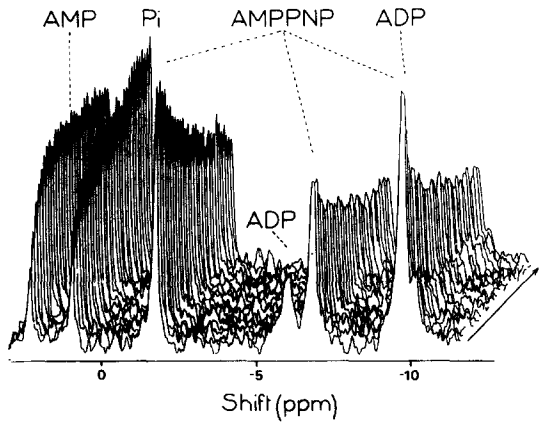

Fig. 5. A series of sequential ^{31}P-NMR spectra acquired at approx. two hourly intervals over about 48 h from a solution of NEM/Nbf-modified CF_1 (45.6 mg per ml, pH 7.6, 20 °C). The direction of the time axis is shown by the arrow on the right of the spectra. Note the signals from a bound ADP molecule initially present, the slow release of bound nucleotide as AMP/inorganic phosphate and a corresponding slow binding of Ca-AMPPNP. A slow hydrolysis of AMPPNP also occurs with further release of inorganic phosphate, P_i.

beta, which are within the range expected for bound nucleotides with chemical shift anisotropy as the dominant source of relaxation (see Discussion). The linewidths of the 'invisible' resonances from ATP bound to this form of CF_1 and from ligands that are bound to the active forms of the enzyme must be much greater than this. Denatured NEM/Nbf modified enzyme showed the presence of both ADP and ATP (Fig. 3).

In contrast to the active forms of CF_1, addition of Ca-AMPPNP to the NEM/Nbf modified enzyme resulted in a slow release of both bound nucleotides (ADP and ATP), in the form of AMP, over about 24 h (Fig. 4), which could be followed by ^{31}P-NMR (Fig. 5). Assuming a first-order process, the half-life for the nucleotide dissociation was estimated to be 4.2 ± 0.8 h at 20 °C and pH 7.6.

In addition to the slow release of nucleotide from NEM/Nbf-labelled CF_1, there was a time-dependent decrease in the intensity of the ^{31}P-NMR signals from the added Ca-AMPPNP, which was attributed to its slow binding to the enzyme (Fig. 5). The fall in free Ca-AMPPNP was consistent with binding at a single site on NEM/Nbf modified CF_1. This site is probably equivalent to the one occupied by the ATP analogue on latent, trypsin-activated and NEM-modified CF_1, as, in common with those forms, no ^{31}P-NMR signals characteristic of bound Ca-AMPPNP could be observed.

Prolonged incubation of the NEM/Nbf modified enzyme at ambient temperature and pH 7.6 resulted in a change in the absorption spectrum consistent with the transfer of the Nbf group from the tyrosine residue [33]. Interestingly, this migration was apparently to both cysteine and lysine residues, in contrast with the mitochondrial enzyme where intramolecular transfer of the Nbf group to a lysine only is well documented [3–7]. Under identical experimental conditions, the loss of the Nbf from tyrosine 328 had a half-life of 5.3 ± 1.2 h, compared to 4.2 ± 0.8 h for the release of bound nucleotide. Thus, it is difficult to distinguish whether the release of adenine nucleotide from the enzyme occurred before or after migration of the Nbf group. The migration was not accompanied by any reactivation.

Discussion

Modification of CF_1 by Nbf-Cl is less easy to characterise than the related reaction of the mitochondrial enzyme. The difficulty arises because there are thiol groups in the CF_1 preparation that are able to react with Nbf-Cl. Latent CF_1 has two surface cysteine residues which react with maleimides or iodoacetamide [22,24,39–41], and they would be expected to react with a nucleophilic reagent like Nbf-Cl. Prior modification of latent CF_1 with Nbf-Cl substantially reduces the number of thiol groups that can be labelled by NEM or *N*-(3-pyrene) maleimide [42]. In this study it was found

that the reactive cysteines could not be entirely blocked by pretreatment of CF_1 with NEM, which suggests that Nbf-Cl has greater access to buried residues because it is more hydrophobic. The reactive thiol groups in the present CF_1 preparation are not all located on CF_1, since peptide purification and sequencing clearly showed that at least one cysteine on contaminating RUBISCO was modified by Nbf-Cl. However, it is estimated that most of the Nbf thiol labelling (greater than 75%) does occur on CF_1 despite the failure to identify a major peptide so labelled. Thus, although the substantial loss of activity observed upon reaction of CF_1 with Nbf-Cl may primarily be attributed to derivatisation of tyrosine 328 on a beta subunit, effects consequent upon modification of thiols cannot be completely excluded. Clearly it is important to show that tyrosine 328 is the only significant site of Nbf-Cl modification on CF_1 in experiments which use the Nbf group as an energy acceptor in resonance energy transfer measurements [40,43,44].

The failure to observe ^{31}P-NMR signals from nucleotides bound to various forms of CF_1 is intriguing. It is known that in ^{31}P-NMR the linewidths of resonances from slowly tumbling molecules are usually dominated by chemical shift anisotropy [45]. Assuming reasonable values of rotational correlation time for CF_1 and the values of Shriver and Sykes [46] for the anisotropy tensor, estimates of about 100 Hz can be made for the expected linewidth. This is close to that observed for the ADP molecule bound to the NEM/Nbf-modified enzyme. This is also consistent with the linewidths of about 30 Hz reported for ADP and AMPPNP bound to myosin subfragment-1 ($M_r = 115\,000$) [46,47]. Recently, however, B.A. Levine (private communication) has shown that the ^{31}P-linewidths of nucleotides bound to native myosin subfragment-1 are about 160 Hz rather than 30 Hz. The reason for this increase of a factor of about 5 over the calculated value is still unclear, but if the same kind of anisotropy exists for nucleotides bound to CF_1 they could have ^{31}P-NMR signals with linewidths over 500 Hz. This would be enough to make the resonances 'invisible'. This raises the interesting possibility that there may be an unusual environment or strain at a single tight nucleotide binding site on both CF_1 and myosin.

Investigations of nucleotide binding to CF_1 have shown that there are up to six nucleotide binding sites on the enzyme [37,48,49]. Studies with the ATP analogue, AMPPNP, are less well characterised. One previous study of AMPPNP binding to CF_1, using radioactively labelled nucleotide and conditions similar to that used here, concluded that there was only one binding site with a dissociation constant of less than 10 μM [50], although another study found two such sites in the presence of divalent metal ions [27]. In both studies other weaker sites were also observed. The NMR method used here, which solely depends on a plot of intensity against molar ratio (Fig. 2), would not be very effective in detecting weak binding especially when ^{31}P-NMR is used. One might expect ^{1}H-NMR to be better at detecting weak binding, since the intensity of the ^{1}H-NMR signals observed in the spin-echo experiments should be very sensitive to any decrease in the spin–spin relaxation time caused by binding to CF_1. Our ^{1}H-NMR results, and those of Roux-Fromy et al. [29], detected only one binding site. Roux-Fromy et al. [29] sought to account for their results by suggesting that two of the sites on CF_1 are blocked by tightly bound, non-exchangeable nucleotides, but the denaturation experiments here have shown that this is not the case. Thus all the NMR studies with AMPPNP only identify a single tight Ca-AMPPNP binding site on CF_1, although this is not in agreement with the radioactive tracer studies (e.g., Ref. 27).

The presence of an Nbf group on tyrosine 328 of a beta subunit and on some unidentified cysteines causes CF_1 to retain an additional ADP molecule during gel filtration. This ADP appears to be bound to a site distinct from the NMR-invisible site, since its ^{31}P-NMR signals have much narrower linewidths. The presence of this ADP is tentatively attributed to the tyrosine modification becuase loss of the Nbf group from this residue can be correlated with the release of both the ADP and ATP from the enzyme in the presence of Ca-AMPPNP.

The reason why modification of a single beta chain tyrosine residue by Nbf-Cl (311 in mitochondrial; 328 in thylakoids) inactivates the proton translocating ATP synthases is not yet known. Measurements of the rate of hydrolysis of stoichiometric amounts of ATP and the release of the product ADP suggested that the presence of the aromatic nitrobenzofurazan ring might drastically attenuate the release of ADP from the modified mitochondrial ATPase [8]. A similar explanation could also apply to the early observations of Ferguson et al. [51] who showed that a stable conformational state, that was transient in the case of the unmodified enzyme, was formed following addition of ATP to the nitrobenzofurazan-derivatised enzyme, as well as to the finding that ADP was released only slowly during steady-state hydrolysis by the modified enzyme [52]. The presence of the additional bound ADP on the derivatised enzyme, as observed in the present work, and the correlation between loss of this group and loss of the Nbf ligand are consistent with findings for the mitochondrial enzyme. In all cases distinction between either the modification directly enhancing the affinity for ADP at a particular site, or the modification interfering with cooperativity between subunits and thus retarding the release of ADP, cannot be readily made. However, the finding that binding of Ca-AMPPNP to the modified enzyme occurs only in association with both loss of the nitrobenzofurazan group from the tyrosine residue and release of nucleotide, implies that the modification in-

terferes with interactions [1] between nucleotide binding sites. Such interactions would also allow the filling of a vacant site on unmodified CF_1 by Ca-AMPPNP to promote the release of previously very tightly bound nucleotide from the site which is invisible by NMR.

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