Characterisation of phosphate binding to mitochondrial and bacterial membrane-bound ATP synthase by studies of inhibition with 4-chloro-7-nitrobenzofurazan

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The effect of phosphate on the inhibition by 4-chloro-7-nitrobenzofurazan of the ATPase activity of the proton-translocating ATP synthase in heart submitochondrial particles was investigated. Binding of phosphate protected strongly against the inhibition. A dissociation constant of 0.2 mM was determined for the enzyme · P_i complex and shown to be independent of pH in the range 7.0–8.0. The protective effect of phosphate was mimicked by arsenate but not by sulphate or malonate. Similar results were obtained for the enzyme from *Paracoccus denitrificans*. 2,4-Dinitrophenol enhanced phosphate binding to the mitochondrial enzyme since the protective effect of phosphate was increased. The data are compatible with protection arising from binding of phosphate to a catalytic site.

ATP synthase *Phosphate binding* *Chemical modification* *Tyrosine residue*

1. INTRODUCTION

The reaction between Tyr-311 of one of the 3 β-chains in bovine heart mitochondrial ATP synthase (but often called ATPase) and 4-chloro-7-nitrobenzofurazan (Nbf-Cl) causes inactivation of the enzyme [1–5]. A similar chemical modification is observed with the corresponding enzymes from bacteria [6–9] and thylakoids [10], which indicates an apparently essential role of this tyrosine residue in the mechanism of the enzyme. Nevertheless, its function is still unclear. In the original work it was reported that substrates of the enzyme, including ATP, ADP and P_i, gave weak or even no protection against modification of the enzyme that had been released from the membrane (F_1 ATPase) [2], thus suggesting that the tyrosine may not be at an active site.

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There have been subsequent reports that P_i can protect the enzyme from modification by Nbf-Cl. First, Ting and Wang [11] found that phosphate protected glycerol-treated soluble F_1 ATPase against inhibition by Nbf-Cl. However, this treatment can lead to depletion of bound nucleotides [12], with the appearance of an increased and non-saturable P_i binding [13]. One of these effects of glycerol could in principle be the basis for the difference [2,11] in the reported protective effect of phosphate, but this possibility has not been investigated. In a more recent study, Cortez et al. [14] showed that the H^+-translocating ATPase from *Rhodospirillum rubrum* was protected from reaction with Nbf-Cl by phosphate both when the enzyme was attached to the membranes of chromatophores, and after purification. In contrast to Ting and Wang, Cortez et al. considered that their data did not implicate phosphate binding at an active site to be responsible for protection of the ATPase against inhibition by Nbf-Cl.

Here, we report that in its membrane-bound state bovine heart mitochondrial ATPase can be

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protected by phosphate from modification by Nbf-Cl. The characteristics of this protection are compared with previous observations on phosphate binding to the ATPase [12], and are consistent with the binding being at a catalytic site. We also report that similar results are obtained with the ATPase of inside-out membrane vesicles from *Paracoccus denitrificans*.

2. MATERIALS AND METHODS

Mg-ATP bovine heart submitochondrial particles were prepared according to the procedure for type-II particles given in [15]. They were resuspended and stored in a buffer containing 0.25 M sucrose, 50 mM triethanolamine HCl, the pH being adjusted to 7.0, 7.5 or 8.0 with KOH. Protein was determined by the Bio-Rad assay which employes a dye-binding method using Coomassie blue [16]. Inverted membrane vesicles from *P. denitrificans* were obtained as in [17]. In this case, protein was determined by the method of Lowry et al. [18].

ATPase activity of submitochondrial particles was measured using a steady-state coupled assay with pyruvate kinase-lactate dehydrogenase as in [11]. The assays were carried out in a Unicam SP 1800 spectrophotometer, following the decrease in absorbance at 340 nm, at a temperature of 30°C, in a total volume of 3.0 ml containing 0.25 M sucrose, 50.0 mM triethanolamine/HCl, 10.0 mM magnesium acetate, 5.0 mM potassium sulphate, 5.0 mM ATP, 1.0 mM phospho*enol*-pyruvate, 0.15 mM NADH and 50 μg of both pyruvate kinase and lactate dehydrogenase (ammonium sulphate suspensions), at pH 8.0. The necessary amount of rotenone and potassium cyanide to inhibit the respiratory chain of the submitochondrial particles, worked out by preliminary titration, was also present. The assays were started by addition of submitochondrial particles to the cuvette. Inactivation by Nbf-Cl was achieved by incubating the particles with the inhibitor, at 30°C in the dark, using the resuspension buffer at one of the chosen pH values mentioned above, in a total volume of 0.4 ml containing between 0.38 and 0.46 mg protein. 20-μl aliquots were taken at intervals for ATPase assay. The submitochondrial particles were preincubated for 60 min before addition of Nbf-Cl in the presence of phosphate or other ligands, to ensure their full binding to the enzyme. Continued Nbf-Cl inhibition during the ATPase assay was avoided because of the 150-fold dilution in the concentration of the inhibitor which followed addition of 20-μl aliquots to the assay mixture. The specific activity of the ATPase activity in the submitochondrial particles was between 4.2 and 5.4 μmol/min per mg protein.

ATPase activity of inside-out membrane vesicles from *P. denitrificans* was assayed by continuous determination of phosphate release in a Technicon autoanalyser as in [1–3].

3. RESULTS

The reaction of Tyr-311 of soluble F_1 mitochondrial ATPase with Nbf-Cl follows pseudo-first-order kinetics throughout the entire reaction period [1,2]. In previous work it has been shown that inhibition by Nbf-Cl of the membrane-bound enzyme of submitochondrial particles proceeded with approximately similar kinetics [2]. The present study was concerned with possible variations of the kinetics of inhibition which derive from the presence of ligands of the enzyme. Consequently, it was necessary to study the kinetics of inactivation of ATPase activity of submitochondrial particles in more detail. It was found that the loss of activity did not follow exact pseudo-first-order kinetics, but deviated from the exponential gradually. This effect was appreciable when the activity had declined to approx. 60% of the original. This is attributed to the presence of other nucleophiles in the submitochondrial particles, apart from the tyrosine of the ATPase, which progressively reacted with Nbf-Cl, decreasing the concentration of the inhibitor, and hence the value of the pseudo-first-order rate constant, k'. In fact, the suspension of particles incubated with Nbf-Cl gradually became yellow-coloured, presumably owing to reaction with available cysteines [19]. It was also observed that the ATPase activity of submitochondrial particles, as measured by the coupled assay procedure, was resistant to inhibition of more than 85–90% (depending on the preparation of particles used) by Nbf-Cl. The residual activity was not inhibited by either venturicidin or a further addition of 200 μM Nbf-Cl. It was therefore concluded that this residual activity was not due to

the F_0F_1-ATPase. Consequently, in calculating results this activity was subtracted from the activity measured at any given time. In addition, as logarithmic plots of the results were not linear, a calculation procedure was used in order to work out the value of the pseudo-first-order rate constant (k') for each inhibition reaction. Assuming that the progressive decrease in the apparent value of k' was due to the reaction of Nbf-Cl with additional nucleophilic groups in the submitochondrial particles which reduced gradually the concentration of the inhibitor in the incubation mixture, it was possible to estimate the time dependence for this reagent depletion and hence also for the value of k'. This made it possible to determine accurately the initial value of k' and then to use this value in calculations of phosphate binding.

When submitochondrial particles were incubated with Nbf-Cl at pH 8.0, the rate at which ATPase activity declined was decreased progressively as the phosphate concentration was increased from zero, through 2.5 to 20.0 mM (fig.1). Phosphate clearly protected the enzyme from inhibition, which could be reversed by inclusion of dithiothreitol in the assay mixture. This reversal is diagnostic of Nbf-modification of tyrosine [2]. A systematic variation of phosphate concentration was also performed, and the results were analysed according to the method of Scrutton and Utter [20]. Fig.2A shows that a plot of k'_{P_i}/k'_0 vs $(1 - k'_{P_i}/k'_0)[P_i]^{-1}$ was linear (k'_0 and k'_{P_i} are pseudo-first-order rate constants for the inactivation by Nbf-Cl in the absence and presence of P_i respectively). The plot did not have an intercept at the origin which meant that the rate constant for the reaction of the enzyme-P_i complex with Nbf-Cl was not zero, although the protective effect of P_i against this reagent was clearly strong (fig.1). From the plot shown in fig.2A, a dissociation constant of 0.2 mM can be calculated for the enzyme·P_i complex.

In his studies of the binding of P_i to F_1, Penefsky found that phosphate binding increased at lower values of pH [21], a result that was interpreted to indicate that the mono-anion form of P_i ($H_2PO_4^-$) was binding. However, the protective effect of P_i upon the reaction of the ATPase of submitochondrial particles with Nbf-Cl was found to be essentially the same as at pH 8.0 (fig.2A) when the experiments were repeated at pH 7.5 (fig.2B)

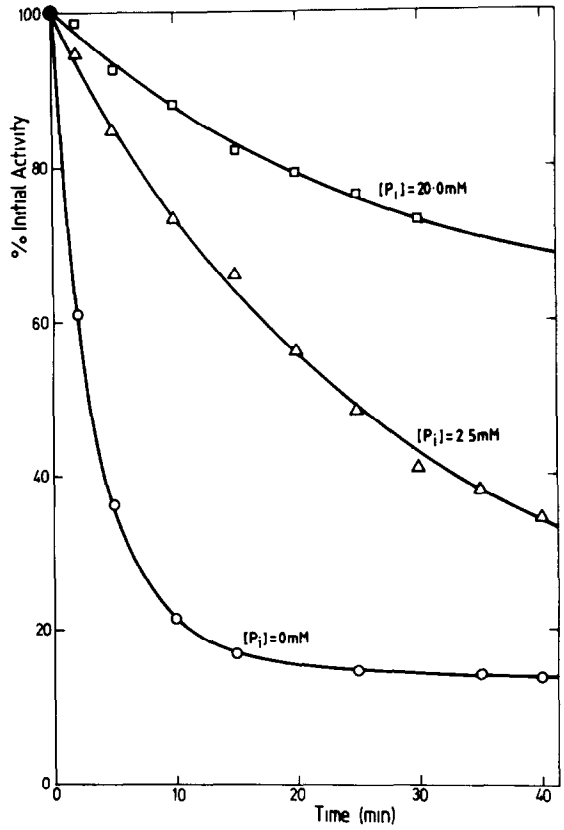


Fig.1. Protection by phosphate against reaction of the ATPase of submitochondrial particles with Nbf-Cl at pH 8.0. The particles were incubated in the presence of 0.1 mM Nbf-Cl together with the indicated concentrations of P_i. The ATPase activity was determined as indicated.

and pH 7.0 (fig.2C). Thus, binding of P_i to the site on the membrane-bound enzyme that influences the reaction with Nbf-Cl does not apparently discriminate between the mono- and di-anion forms of phosphate.

The protective effect of P_i illustrated by figs 1 and 2 was mimicked by arsenate, although its dissociation constant from the enzyme was 3-fold greater than for P_i (fig.3), but not by either sulphate or malonate. Thus, this binding site appears to be specific for phosphate or its analogue arsenate, and is not a non-specific anion-binding site. The question arises as to whether this site is catalytic or regulatory. In this context, it is striking that the phosphate protection reported here is similar to that reported for the enzyme from *R.*

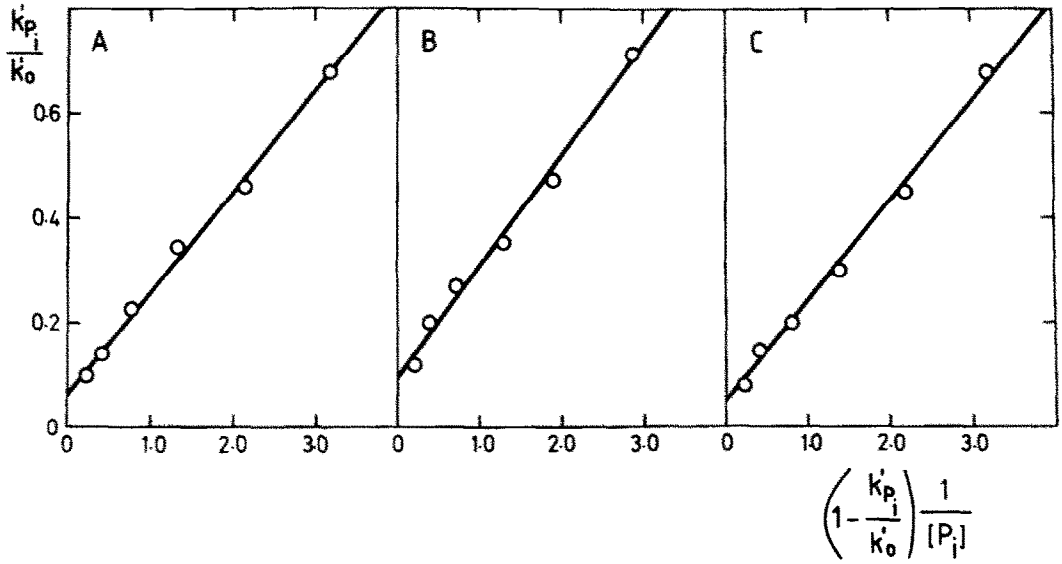
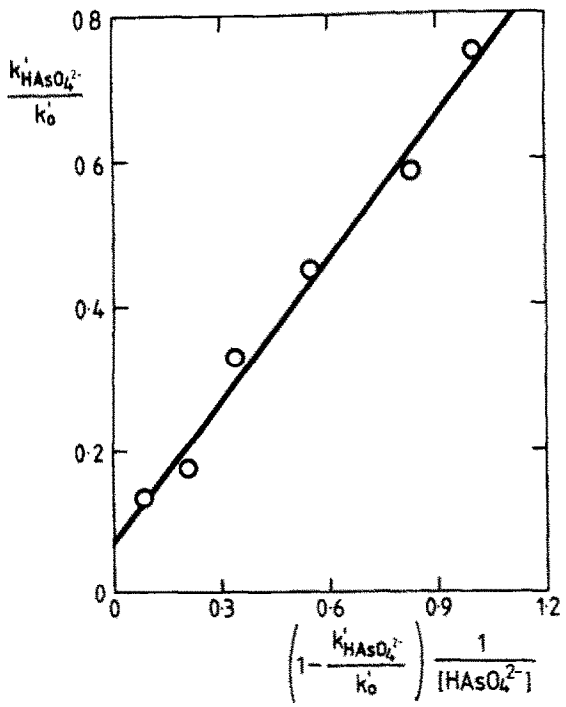


Fig.2. Determination of the dissociation constant (K_p) for P_i binding to the F_1 ATPase of submitochondrial particles at different pH values. The particles were incubated in the presence of 50 μM Nbf-Cl at pH 8.0 (A), 100 μM Nbf-Cl at pH 7.5 (B) and 200 μM Nbf-Cl at pH 7.0 (C) with the range of P_i concentrations 0, 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 mM in each case. A different concentration of Nbf-Cl was used for each pH in order to obtain similar rates of inhibition [2]. The ATPase activity was measured at intervals of 5 min until 40 min after the addition of Nbf-Cl to the particles. The residual ATPase activity, resistant to inhibition by Nbf-Cl, was determined by following the inhibition reaction in the absence of phosphate until 90 min after the start. The values of K_p obtained from the slopes of each plot are 0.19 mM at pH 8.0 (A), 0.22 mM at pH 7.5 (B) and 0.19 mM at pH 7.0 (C).



rubrum [14]. Furthermore, we have observed that the concentrations of phosphate and arsenate indicated in figs 1–3 also protect the ATP synthase of *P. denitrificans* from inactivation by Nbf-Cl [6]. In these experiments the latency [6] of the ATPase activity of this enzyme was overcome by including sulphite, an activating anion (A.J. Greenfield and S.J. Ferguson, unpublished) in the assay medium. Thus, a phosphate-binding site of similar affinity has now been detected for two bacterial enzymes as well as for the mitochondrial enzyme. A catalytic role for the site is the more plausible because it is less likely that a regulatory site with similar

Fig.3. Determination of the dissociation constant for arsenate binding to the F_1 ATPase of submitochondrial particles at pH 7.5. The particles were incubated at 30°C in the dark in the presence of 0.1 mM Nbf-Cl at a range of concentrations of arsenate, viz. 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 10.0 mM. The ATPase activity was measured at regular times as described in fig.2. The value of K_d [enzyme·arsenate] obtained from the slope is 0.66 mM.

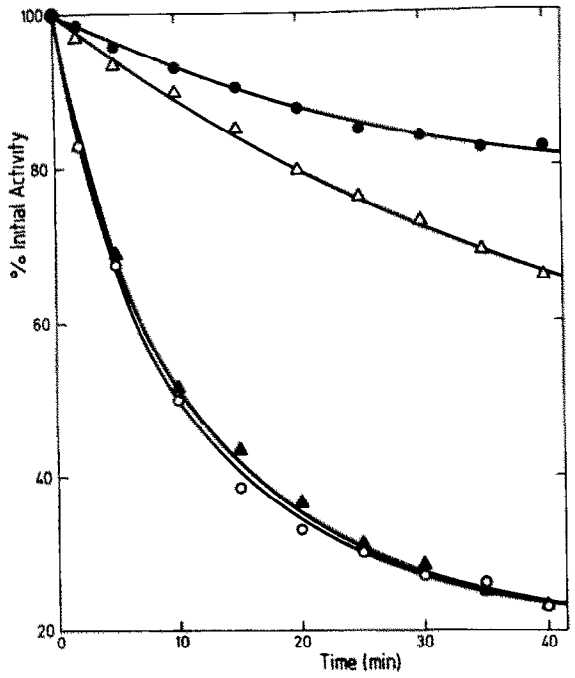


Fig.4. Effect of DNP on the protection by phosphate against reaction of the ATPase of submitochondrial particles with Nbf-Cl at pH 7.5. The particles were incubated in the presence of 0.1 mM Nbf-Cl and either 0 mM (○) or 5.0 mM phosphate (△) at 30°C in the dark. Alternatively, 0.2 mM DNP was also present together with 0.1 mM Nbf-Cl and either 0 mM (▲) or 5.0 mM (●) phosphate. The ATPase activity was determined at intervals of 5 min as shown. The weak absorbance of the DNP that was carried over into the spectrophotometer cuvette for assay of ATPase was compensated by inclusion of a similar concentration of DNP in the reference cuvette.

characteristics would be found in both bacterial and mitochondrial enzymes.

Penefsky reported that 2,4-dinitrophenol (DNP) enhanced binding of phosphate to purified F_1 ATPase [13]. A similar effect was observed when the effect of P_i upon protection against reaction with Nbf-Cl was studied in the presence of DNP (fig.4). Experiments similar to those of fig.2, but using P_i concentrations between 0.05 and 1 mM, showed that at 0.2 mM DNP the dissociation constant for phosphate from the enzyme decreased to 0.1 mM from the value of 0.2 mM observed in the absence of DNP (fig.2). Control experiments showed that DNP neither reacted with Nbf-Cl nor influenced the kinetics of inhibition of the ATPase by Nbf-Cl in the absence of phosphate (fig.4).

4. DISCUSSION

The use of the present results is that they characterise the binding of phosphate to the membrane-bound ATP synthase. The value (0.2 mM) determined here for the dissociation constant of the enzyme·P_i complex bears a striking similarity to the constant determined for phosphate with the reconstitutively active subunit removed from the *R. rubrum* enzyme [22], as well as with the membrane-bound enzyme from this bacterium [14]. The K_m value for P_i in ATP synthases from different sources [23,24] is also approx. 0.2 mM, which could suggest that the phosphate-binding site that affects modification with Nbf-Cl is a catalytic site.

On the basis of their work with glycerol-treated mitochondrial F_1 ATPase, Ting and Wang [11] also concluded that phosphate binding to a catalytic site was responsible for protection against Nbf-Cl, although compared with the present work a higher dissociation constant of 1.3 mM was determined. In later work [25] a dissociation constant of 0.43 mM for phosphate was estimated when Mg^{2+} was present. Cortez et al. [14], working with both solubilised and membrane-bound F_1 ATPase from *R. rubrum*, concluded that both the tyrosine group labelled by Nbf-Cl and P_i binding that gave protection against Nbf-Cl were not located at a catalytic site. This view was based upon the observation that ATP was not able to protect to the same extent as P_i against modification by Nbf-Cl. However, the phosphate binding was not characterised by comparison with analogues or by observing the effect of a known effector upon the interaction of P_i with a presumed catalytic site on the enzyme [13]. Our results with arsenate (fig.3) or DNP (fig.4) indicate a strong similarity between a previously characterised P_i site and the site at which P_i can protect against Nbf-Cl.

Binding of phosphate that results in protection against Nbf-Cl is not influenced by pH changes from 7.0 to 8.0. This result contrasts with the report that phosphate binding to the soluble F_1 ATPase decreases markedly upon raising the pH from 7.0 to 8.0 [13,21]. The latter result was taken as an indication that the enzyme preferentially binds the mono-anion form of phosphate. However, the fact that a similar pH dependence

for phosphate binding to the isolated β-subunit of the enzyme from *R. rubrum* is significantly affected by raising the concentration of Mg^{2+} suggests that interpretation of the pH dependence of phosphate binding will require further study. Our experiments were done in the absence of added Mg^{2+} but it will be of value in future work to investigate whether the enzyme has retained tightly bound Mg^{2+} acquired during the isolation procedure.

It is possible to suggest why the protective effect of P_i was not observed in the original work on the reaction of Nbf-Cl with soluble mitochondrial ATPase, in contrast to the present data with submitochondrial particles. Protection was tested in the presence and absence of added ADP [2]. It is clear from the work of Ting and Wang [11] and from our own unpublished observations that ADP antagonises the protective effect of P_i. We suspect that the sample of enzyme tested in the absence of added ADP [2] must have had sufficient ADP bound to mask the protective effect of P_i. We have now observed protection of soluble F_1 ATPase by phosphate under conditions in which ADP binding to the enzyme is minimised. Further details of these experiments will be given in a subsequent publication. There is now no reason to believe that glycerol treatment of F_1 ATPase substantially alters the protective effect of P_i towards modification by Nbf-Cl. A role for Tyr-311 of a β-chain at or near a catalytic binding site for P_i warrants serious consideration.

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