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ON THE ROLE OF THE ESSENTIAL TYROSINE RESIDUE IN THE MITOCHONDRIAL ATPase

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Summary

Aurovertin has been used as a probe for the properties of an inactive derivative of bovine heart mitochondrial ATPase which was modified on just one tyrosine residue. It is suggested that this modification inhibits the enzyme by preventing a step subsequent to a conformational change produced by addition of ATP to the enzyme.

Although the mitochondrial ATPase is a large multi-subunit enzyme, activity is totally abolished when just one tyrosine residue is modified by 4-chloro-7-nitrobenzofurazan (Nbf-Cl)[1,2]. It is now important to elucidate the role of this tyrosine residue which probably lies on the surface of the enzyme and has a normal pK_a [2]. As k_{cat} of the ATPase is constant in the pH range 6.25 to 9.2, this tyrosine could participate in a rate determining proton donation to ATP [3], but the absence of marked protection of the tyrosine by substrates [2] may be an indication that this residue is not at an active site. The antibiotic aurovertin can be used as a fluorescent probe for conformational states of the ATPase [4,5], and this report is concerned with the use of aurovertin to show how modification of this tyrosine alters some aspects of the conformational behaviour of the enzyme.

Mitochondrial ATPase was prepared by the method of Knowles and Penefsky [6] and modified on tyrosine by Nbf-Cl as described before [2]. Aurovertin was from a batch prepared by Dr. R.M. Bertina [7]. For fluorescence measurements an ammonium sulphate precipitate of the Nbf-ATPase was freed from salts by passage through a Sephadex G-25 column (medium), equilibrated with a buffer containing 50mM triethanolamine hydrochloride

Abbreviations: Nbf-Cl, 4-chloro-7-nitrobenzofurazan (formerly NBD-Cl); Nbf-ATPase, ATPase modified on tyrosine by Nbf-Cl.

and 100 mM KCl at pH 7.5. Fluorescence was measured in a Perkin Elmer MPF2A fluorimeter using slit widths of 6 nm and excitation and emission wavelengths of 370 nm and 470 nm, respectively.

The essential features of our observations are shown in Figs. 1a and 1b. The fluorescence intensity of aurovertin in the presence of Nbf-ATPase is quenched upon adding ATP, as briefly reported before [2], but subsequent addition of Mg^{2+} has no effect on this intensity which remains constant for twenty minutes, the longest period examined (Fig. 1a). Introduction of dithiothreitol causes a slow increase in aurovertin fluorescence to a level which is to be expected when the ligands of the ATPase are ADP, phosphate and Mg^{2+} (see ref. 5). The time course of this increase in fluorescence corresponds closely to the rate at which dithiothreitol can remove the nitrobenzofurazan group from the enzyme [2]. The exact kinetics are difficult to measure because of the photobleaching effect reported before [5]. However the rate is dependent on the dithiothreitol concentration which supports the contention that the fluorescence increase reflects regeneration of the native enzyme. Estimated half times for this change are, at 1 mM dithiothreitol 45 s, and at 0.5 mM 90 s.

In contrast the addition of Mg^{2+} after ATP to the unmodified enzyme produces a different response from aurovertin. Fig. 1b shows that if dithio-

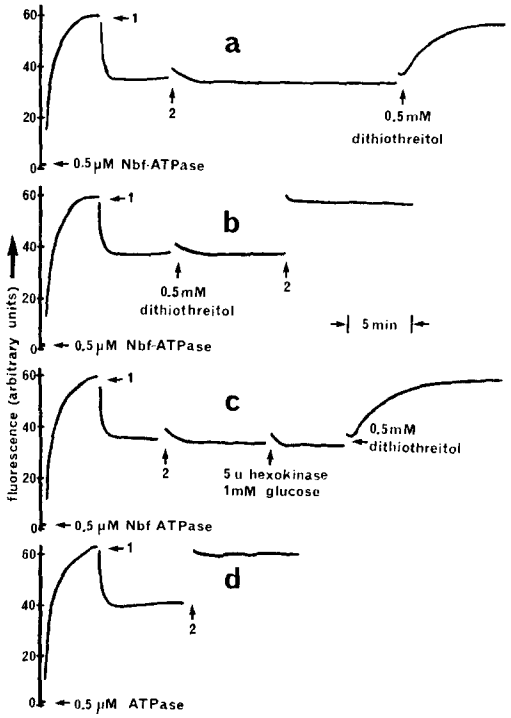

Fig.1. Effect of ATP and Mg^{2+} on aurovertin fluorescence in the presence of Nbf-ATPase or native ATPase. Enzyme was added as indicated to 1 μM aurovertin in 2 ml 50 mM triethanolamine hydrochloride, 100 mM KCl, pH 7.5 at 25°C. At 1 and 2 0.2 mM $MgCl_2$ were added respectively. Other additions were as shown.

threitol is added to a solution of modified enzyme, thus regenerating native ATPase, in the presence of ATP then subsequent addition of Mg^{2+} produces an immediate rise in aurovertin fluorescence to a level which is again characteristic of the ligands being ADP, phosphate and Mg^{2+}. Indeed it can be seen from Fig. 1b that the final fluorescence level in this experiment is very similar to that seen in Fig. 1a after the addition of dithiothreitol. As a control it is shown in Fig, 1d that addition of ATP to a sample of enzyme which had not been reacted with Nbf-Cl produced an aurovertin fluorescence quenching which was also reversed when Mg^{2+} was added.

At first sight the data reported here would be consistent with the Nbf-ATPase binding ATP, or ATP and Mg^{2+}, when the aurovertin fluorescence was quenched, as ATP is well known to be effective at reducing the fluorescence intensity of the enzyme-aurovertin complex [4,5]. However, such an interpretation may be an oversimplification for the following reasons. Analysis of a solution of Nbf-ATPase (0.5 μM) to which 0.2 mM ATP and Mg^{2+} had been added showed that essentially all the added ATP had been hydrolyzed as judged by the inorganic phosphate released. This is no unexpected since a sample of Nbf-ATPase will always contain some active enzyme with sufficient catalytic capacity to hydrolyse the added ATP, especially in the presence of Mg^{2+}. Moreover as indicated in Fig. 1c addition of hexokinase and glucose to remove any ATP in solution did not reverse the aurovertin fluorescence quenching in the period examined which was up to 5 min. Hence in a situation where there should be ADP but no ATP in solution, the aurovertin fluorescence remains quenched to an extent which would, by comparison with previous experiments [4,5], suggest that ATP is bound to the enzyme. As a control experiment ATP and then Mg^{2+} were added to the Nbf-ATPase before aurovertin. The antibiotic fluorescence was again enhanced upon adding dithiothreitol to the same extent as was seen in the experiments illustrated in Figs. 1a and 1c. This indicated that the aurovertin fluorescence intensity was acting as a probe for the state of the enzyme produced by addition of ATP and Mg^{2+}, and that the inhibitory action of aurovertin on ATPase activity [4] did not influence this state.

The observations presented in Figs. 1a and 1c would be consistent with a very tight binding of ATP to Nbf-ATPase such that even hexokinase might not be able to convert all the added ATP to ADP. However, several further experiments militate against this proposal. When stoichiometric quantities of Nbf-ATPase (20 μM) and [^{3}H]ATP were mixed together with 20 μM Mg^{2+}, a subsequent gel filtration on a small Sephadex G-25 column (fine, 10 × 0.5 cm) the enzyme fraction contained only one sixth of the added counts which was the same proportion that was found in a sample of the unmodified enzyme after a similar experiment. From this experiment it does not appear that the Nbf-ATPase forms an especially tight complex with ATP. A second approach involved adding 20 μM ATP and Mg^{2+} to an equal amount of Nbf-ATPase. That ATP did not become tightly bound to the enzyme under these conditions was indicated by the finding that all the added ATP was hydrolysed, presumably by the contaminating active ATPase which was inevitably present.

The stability of the species responsible for the quenched fluorescence after addition of ATP and Mg^{2+} to the Nbf-ATPase was investigated in the

following way. 0.8 mM ATP and Mg^{2+} were added to 0.5 ml of Nbf-ATPase, and after five minutes this solution was passed down a Sephadex G-25 column equilibrated with the triethanolamine hydrochloride/KCl buffer described in the legend to Fig. 1. When the enzyme collected from this column was added to aurovertin the usual fluorescence was observed which was quenched in the normal way by adding ATP. This suggests that the form of the modified ATPase responsible for the quenched aurovertin fluorescence is not stable in the absence of ligands in solution.

If a quenching of aurovertin fluorescence is simply indicative of ATP binding to mitochondrial ATPase, then the observations shown in Fig. 1a require very tight binding of added ATP, at least in the presence of Mg^{2+}, so that the aurovertin fluorescence remains quenched even when the predominant ligand is ADP. Experiments reported in this paper have not detected sufficiently tight binding of added ATP to be readily consistent with this explanation. Penefsky's finding [8], which we have also confirmed, that the non-hydrolysable ATP analogue, adenylyl imidodiphosphate does not quench aurovertin fluorescence although it is a strong competitive inhibitor of ATPase activity [8,9] is also relevant. Adenylyl imidodiphosphate is a very close structural analogue of ATP [10], and in view of its high affinity for the ATPase, aurovertin fluorescence quenching, if simply a reflection of ATP binding, might be expected on adding this inhibitor to the ATPase. It is worth noting that in muscle adenylyl imidodiphosphate has been shown to be close to ATP in its effectiveness at dissociating actomyosin at high ionic strength [11]. Taken together, the observations with the inactive enzyme and adenylyl imidodiphosphate suggest that consideration of the possibility that aurovertin fluorescence quenching represents something more than ATP binding is warranted. The increase in the number of binding sites for aurovertin upon adding ATP [4] may indicate subunit-subunit interactions, and it is not inconceivable that the quenching of aurovertin fluorescence relies on the binding of more than one ligand to the subunits of the enzyme, or perhaps from a step in the reaction pathway beyond ATP binding.

It seems from Fig. 1 that, upon addition of ATP and Mg^{2+}, Nbf-ATPase can adopt the conformation that is characterised by quenched aurovertin fluorescence, but that relaxation of this conformational state can only occur when the nitrobenzofurazan group is removed from the enzyme by dithiothreitol. The modification of the essential tyrosine residue seems to prevent a step in the catalytic mechanism which may be subsequent to ATP binding to the enzyme, so that Nbf-ATPase should permit the study of an intermediate state in the catalytic mechanism.

A schematic explanation of the observations representing a minimal hypothesis is given in Fig. 2. The notation *Nbf-ATPase refers to a conforma-

$$\text{Nbf-ATPase} \xrightarrow[(2)\ Mg^{2+}]{(1)\ ATP} {}^{*}\text{Nbf-ATPase} \begin{bmatrix} ADP \cdot Mg^{2+} \\ ADP \cdot Mg^{2+} \cdot P_i \\ ADP \cdot P_i \end{bmatrix} \xrightarrow{\text{dithiothreitol}} \text{ATPase} \begin{bmatrix} ADP \cdot Mg^{2+} \\ ADP \cdot Mg^{2+} \cdot P_i \\ ADP \cdot P_i \end{bmatrix}$$

Fig. 2. Summary of conclusions. For explanation see text.

tion of the enzyme that is characterised by a quenched aurovertin fluorescence. Species shown in square brackets are possible combinations of bound ligands; at present a decision between these possibilities cannot be made. As sequential addition of 0.2 mM ADP, $MgCl_2$ and phosphate to the Nbf-ATPase did not produce a quenching of aurovertin fluorescence, it appears that *Nbf-ATPase is only produced from added ATP and Mg^{2+}. The role of Mg^{2+} in the conformational behaviour of both the unmodified enzyme and Nbf-ATPase is unclear, and the extent to which this ion is involved in the fluorescence changes is thus uncertain. The data of Fig. 1b and Fig. 1d can be interpreted in terms of added Mg^{2+} producing a rapid completion of catalysis, and hence relaxation of the ATPase to a state characterised by high aurovertin fluorescence.

Aurovertin fluorescence is assumed to be responsive to events at a catalytic centre of the ATPase. There is no direct evidence for this, but the observation that addition of ITP can produce the aurovertin fluorescence quenching [4] at least suggests that adenine nucleotide binding to a regulatory site is not responsible for the changes in fluorescence.

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