AN UNUSUAL AND REVERSIBLE CHEMICAL MODIFICATION OF SOLUBLE BEEF HEART MITOCHONDRIAL ATPase

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1. Introduction

Mitochondrial ATPase is thought to be the terminal ATP synthesising enzyme of oxidative phosphorylation [1] but little is known about its reactivity towards chemical reagents. Yet in principle, a comparison of chemical reactivity of the residues in the soluble and membrane bound enzyme could give valuable information about the location of F_1 in the membrane, and about any groups that may be essential for reconstitution of the enzyme into F_1-depleted particles [2]. Previous work has suggested that sulphydryl groups are not important in the catalytic process [3] and that modification of tyrosine residues leads to loss of activity [4]. This paper describes an unusual reaction between the ATPase from beef heart mitochondria (F_1) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) which is readily reversible and compares this modification with others brought about by more conventional chemical reagents.

2. Materials and methods

NBD-chloride was synthesized by the method of Boulton et al. [5]. Other reagents were obtained from commercial sources and were of the highest purity available. ATPase was assayed by following phosphate release at pH 8.0 in a medium which contained ATP (5 mM), $MgCl_2$ (5 mM) and Tris–maleate (50 mM). Beef heart F_1 was prepared by the method of Knowles and Penefsky [6] and had a specific activity* of 90–115. Chemical modifications were carried out at room temperature by incubation of the enzyme and reagent in triethanolamine hydrochloride (50 mM), sucrose (200 mM) and EDTA (4 mM) at pH 7.5. ATP or ADP (4 mM) were added as appropriate. An exception was the reaction between F_1 and phenylglyoxal (10 mM) which was carried out in *N*-ethyl-morpholine acetate (200 mM) at pH 8.0. Protein concentrations were determined by the method of Lowry [7] and the molecular weight was taken to be 360 000.

3. Results

Incubation of NBD-Cl (100 μM) with F_1 (6 μM) results in essentially total inactivation of ATPase activity within 1 hr. The rate of inactivation follows the increase in absorbance at 385 nm. (fig. 1). A semi-log plot of the absorbance increase shows that the reaction follows pseudo first order kinetics (pseudo first order rate constant = 0.057 min^{-1}). This indicates that only one type of reaction is occurring. The presence of ADP or ATP during the reaction caused no appreciable change in the rate. Precipitation of the enzyme with ammonium sulphate followed by filtration through Sephadex G-25 to remove excess NBD-Cl causes a slight reactivation (1–2% of the original ATPase activity). A spectrum of the NBD-enzyme after this treatment (fig. 2) clearly shows an absorbance maximum at 385 nm.

Addition of dithiothreitol (500 μM) immediately restores the ATPase activity to 100% and is accompanied by the disappearance of the 385 nm absorbance peak and the appearance of a new peak at 328 nm (fig. 2). The new peak does not, however, repre-

* Specific activity refers to micromoles of phosphate liberated per min. per mg protein.

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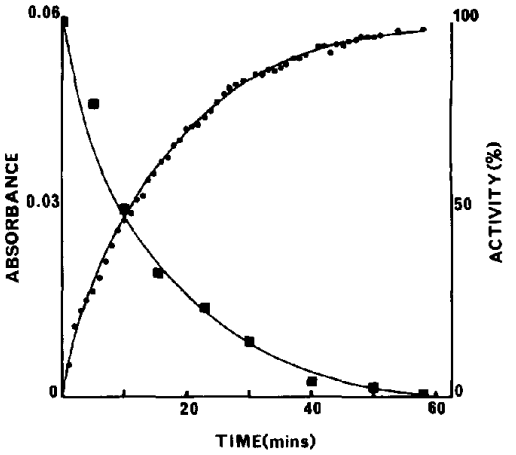

Fig. 1. Increase in absorbance at 385 nm and decrease in ATP-ase activity with time on treatment with NBD-Cl. (▪) Enzyme activity. (•) Absorbance at 385 nM.

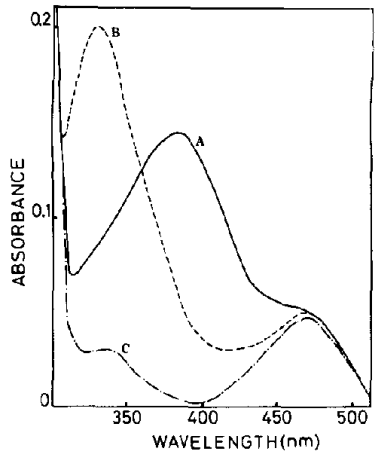

Fig. 2. Absorption spectra of F_1 after reaction with NBD-Cl: (A) ————, after ammonium sulphate precipitation and passage through Sephadex G-25; (B) – – – – – –, (A) following addition of dithiothreitol (500 μM); (C) –.–.–.–.–, (B) after ammonium sulphate precipitation and passage through Sephadex G-25.

sent a dithiothreitol adduct of NBD (λ_{max} = 425 nm). Ammonium sulphate precipitation followed by filtration through Sephadex G-25 removes the 328 nm peak from the protein absorbance spectrum.

By analogy with work on the reaction between NBD-Cl amino acids [8] NBD-Cl would be expected to react with sulphydryl groups under our conditions. The spectral properties of the NBD-F_1 adduct are not consistent with the formation of an S-NBD bond [8] unless the absorption maximum of the adduct has been shifted 35–40 nm. S-NBD labelling of F_1 would not be expected as previous work has implicated neither a reactive nor a catalytically essential sulphydryl group [3]. In support of this, we have detected no change in ATPase activity after prolonged incubation (8 hr) with 100 μM iodoacetamide nor any reaction between F_1 and the thiol reagent 4-aldrothiol.

The F_1-NBD adduct has a fluorescence exitation and emission spectrum which is characteristic of nitrogen-NBD derivatives [9]. Treatment of the adduct with dithiothreitol, followed by ammonium sulphate precipitation and filtration through Sephadex G-25 results in unchanged fluorescence spectra. It must therefore be concluded that the fluorescent properties of NBD-F_1 are due to a small amount of NBD-nitrogen labelling and not associated with the absorbance peak at 385 nm. This non-fluorescent peak at 385 nm may therefore represent an NBD-oxygen bond [9]. No preparative or spectral details of NBD-oxygen derivatives have been published to the knowledge of the authors, but we find that incubation of NBD-Cl with *N*-acetyl tyrosine-ethyl ester at pH 8.0 results in the formation of a new absorbance peak with λ_{max} = 385 nm. This peak disappears rapidly on addition of dithiothreitol. This behaviour is similar to that of the NBD-F_1 species but in the absence of spectral characteristics of other possible NBD-derivatives this similarity can only be suggestive.

Since the identity of the NBD-F_1 product giving rise to the 385 nm peak is not known, the number of groups reacting cannot be directly estimated spectrophotometrically. However, this number can be estimate by measuring the decrease in unreacted NBD-Cl concentration at 345 nm. Allowing for the overlap of the 385 nm absorbance peak and the small amount of NBD-nitrogen labelling, this method indicates that one, or at the most two moles of NBD-Cl react per mole of F_1 to give the inactive NBD-F_1 adduct.

As a further step towards investigating which group(s) on F_1 is attacked by NBD-Cl, we have studied the effects of several other modifing reagents. Reaction of fluorodinitrobenzene (500 μM) with F_1 results in loss of ATPase activity over a period of 1 hr. This inactivation is not reversed by thiolysis which is known to remove the dinitrophenyl group from imidazoles, sulphydryls and phenolic hydroxyl groups

[10]. This suggests that the loss of activity in this case results from reaction with one or more essential amino groups, which is supported by experiments carried out with pyridoxal phosphate, in the hope of introducing an active site-directed fluorescent probe. Pyridoxal phosphate (5 mM) caused 90% inactivation of F_1 in 1 hr but the presence of either ADP or ATP slowed the rate at which activity was lost. Reduction of the pyridoxal phosphate bound to the enzyme with sodium borohydride followed by the measurement of the absorbance of 324 nm [11] showed that about 10 moles of pyridoxal had bound per mole of F_1 and therefore the labelling was non specific.

The inactivation of F_1 by both phenyl-glyoxal and *N*-acetyl-imidazole may implicate essential arginine and tyrosine residues respectively. Treatment with the latter, but not the former reagent severely impedes the reaction of NBD-Cl with F_1. We have not been able to decide whether this last effect is due to the acetylation of the essential amino acid with which NBD-Cl reacts, or to secondary effects of acetylation of a variable number of other groups. All the modifications described in this work yielded a homogeneous band in the ultracentrifuge with 'S' values which were identical with the native enzyme.

4. Discussion

The reaction of NBD-Cl with soluble beef heart mitochondrial ATPase results in a reversible inactivation of the enzyme, and points to the functional role of an unusual nucleophilic species in the activity of the enzyme. The evidence, based on the spectral properties of the reaction product in comparison with the products formed with different amino acids, suggest that the group modified may be tyrosine. Furthermore, our preliminary experiments indicate that F_1 can be similarly inactivated when it forms part of a submitochondrial particle and activity restored by treatment with dithiothreitol. Also when NBD-inactivated F_1 is reconstituted into F_1-depleted submitochondrial particles addition of dithiothreitol restores the ATPase activity. As the inactivating reaction between NBD-Cl and the beef heart enzyme appears to involve only one or perhaps two identical residues, it would be of interest to see whether, for example, the chloroplast or yeast ATPase behaved in a similar manner.

These results also show that NBD-Cl will react with groups other than amino and sulphydryl in proteins to yield a product which is not fluorescent. A careful spectral analysis of the reaction is therefore required to establish the specificity of the reaction with a given enzyme.

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