ATP binding to bovine heart cytochrome *c* oxidase

A photoaffinity labelling study

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ATP influences the kinetic properties of cytochrome *c* oxidase. A photoactivatable radioactive ATP analogue was used to localize the nucleotide-binding site on the bovine heart enzyme. Subunits IV and VIII were specifically labelled, suggesting that these two nuclear-coded polypeptides may play a regulatory role on the oxidase functions.

INTRODUCTION

Cytochrome *c* oxidase, the terminal component of the mitochondrial respiratory chain, catalyses the transfer of electrons from cytochrome *c* to O₂ coupled to the transport of protons across the mitochondrial inner membrane (Azzi, 1980; Capaldi *et al.*, 1983).

Ferguson-Miller *et al.* (1976) for bovine heart cytochrome *c* oxidase and Roberts & Hess (1977) for the yeast enzyme have found that electron transfer from cytochrome *c* to O₂ is inhibited by millimolar concentrations of ATP and have suggested that this polyanion may have a regulatory role on the function of the enzyme *in vivo*. The effect has been ascribed to the binding of ATP to the oxidase rather than to cytochrome *c*.

We have attempted to determine which of the 13 different subunits of bovine heart cytochrome *c* oxidase is involved in binding ATP by using a photoactivatable radioactive derivative of ATP. On illumination this ATP analogue is converted into a highly unstable intermediate able to react with its neighbouring molecules, which can then be identified by their radioactive labelling (Bayley & Knowles, 1977; Bayley, 1983).

MATERIALS AND METHODS

Cytochrome *c* oxidase was isolated from hearts by the method of Steffens & Buse (1976), with omission of the last dialysis step, and stored in small portions at 75 mg/ml in 250 mM-sucrose/10 mM-sodium phosphate buffer, pH 7.5, at –80 °C. Its activity was assayed polarographically at 25 °C, as reported by Vik & Capaldi (1980), in 25 mM-Tris/cacodylate buffer, pH 7.6, containing 0.02% egg lysolcithin (Sigma Chemical Co., St. Louis, MO, U.S.A.) or 0.1% lauroyl maltoside. 8-Azido-[γ -³²P]ATP [tetra(triethylammonium) salt] (33 Ci/mmol) in Tricine was purchased from N.E.N., Dreieich, West Germany.

Cytochrome *c* oxidase was diluted to 1 mg/ml in 25 mM-Tris/cacodylate buffer, pH 7.6, containing 0.1% lauroyl maltoside at 0 °C, and after the addition of various effectors 8-azido-[γ -³²P]ATP (2 × 10⁵ or 2 × 10⁶ c.p.m.) was added under a red safety light. The mixture (250 μ l samples) was illuminated with a long-wave

u.v. lamp (Ultraviolet Products, Saint Gabriel, CA, U.S.A.) for 10 min at 0 °C. Certain samples contained reduced cytochrome *c* (2:1 molar ratio with respect to the oxidase) and 1 mM-NaN₃. The samples were layered on 10% (w/v) sucrose/20 mM-Tris/cacodylate buffer, pH 7.6, and centrifuged at 250 000 *g* for 6 h. The pellets were resuspended and electrophoresed in accordance with Kadenbach *et al.* (1983). The gels were stained with Coomassie Brilliant Blue, destained, dried and exposed to Kodak X-Omat at –80 °C. The amount of radioactivity associated with the gel was determined by gel slicing, dissolution in tissue solubilizer Soluene 350 (Packard) and counting in a Packard Tri-Carb 300 C scintillation counter.

RESULTS AND DISCUSSION

Keilin–Hartree particles were used in previous experiments to show that there are two kinetically distinct sites for cytochrome *c* on bovine heart cytochrome *c* oxidase with apparent *K_m* values 5 × 10^{–8} M and 0.35 × 10^{–6} M for the high-affinity and low-affinity sites respectively and that the high-affinity site disappears in the presence of millimolar concentrations of ATP (Ferguson-Miller *et al.*, 1976).

Fig. 1 shows the biphasic kinetics of the isolated oxidase in the presence of detergents (apparent *K_m* values 8 × 10^{–8} M and 7 × 10^{–6} M for the high-affinity and low-affinity sites respectively) and that again the high-affinity site is abolished by ATP; under these conditions a single site is evident with an apparent *K_m* of 8 × 10^{–7} M. This clear-cut effect of ATP may be related to a yet unknown regulatory role of ATP on the functions of the oxidase *in vivo*.

If so, the presence of (a) specific binding site(s) for ATP on the oxidase is expected. Moreover, ATP should bind to the enzyme even in the presence of ADP and phosphate, since they have a poor effect on the high-affinity cytochrome *c*-binding site (Ferguson-Miller *et al.*, 1976).

8-Azido-ATP, a photoreactive analogue of ATP with very similar binding properties, has been used to map the

Fig. 1. Effect of ATP on the kinetic properties of isolated bovine heart cytochrome *c* oxidase

Cytochrome *c* oxidase samples (25.6 nm-cytochrome aa_3) were assayed in 25 mM-Tris/cacodylate buffer, pH 7.6, containing 7 mM-ascorbate, 0.7 mM-*NNN'*-tetramethylphenylenediamine and 0.02% egg lysolecithin at 25 °C over the horse cytochrome *c* (Sigma type VI) concentration range 0.02–30 μ M in the absence (○) or in the presence of 3 mM-ATP (sodium salt, crystalline; Sigma) (■).

Fig. 2. Labelling of bovine heart cytochrome *c* oxidase with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

Lane 0 shows the Coomassie Blue-staining profile of the enzyme, with the subunit nomenclature of Buse *et al.* (1983). Lanes from 1 to 6 show the autoradiographic patterns of the protein complex labelled with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^5 c.p.m.) in the presence of (1) 10.5 μ M-ATP, (2) 50 mM-sodium phosphate, (3) 10.5 μ M-ADP, (4) no addition, (5) 10.5 μ M-ADP and 50 mM-sodium phosphate, and (6) 0.2% SDS.

ATP-binding site on different enzymes (Wagenvoort *et al.*, 1980; Campbell & MacLennan, 1983; Hollemans *et al.*, 1983). On illumination, its azido group is converted into an intermediate able to cross-link to the amino acid residues forming its binding site on the protein. The subunit(s) involved can then be identified by determining the radioactivity associated with a SDS/polyacrylamide gel of the protein complex after electrophoresis.

Fig. 2 shows the amount of radioactive azido-ATP bound to the different subunits of the oxidase after illumination with long-wave, non-protein-damaging, u.v. radiations. Subunit IV and one of the subunits VIII are selectively labelled (lane 4), suggesting that they are involved in the binding of ATP. This selectivity is even more evident if compared with the pattern of labelling of the enzyme in the presence of the denaturing detergent SDS, where all subunits were modified (lane 6).

Although ATP completely protects from the labelling, the effect of ADP or P_i is much lower (compare lane 1 with lanes 2, 3 and 5 in Fig. 2). Neither the redox state of the enzyme nor the presence of cytochrome *c* appears to influence the extent of labelling by azido-ATP. No binding of azido-ATP was found on cytochrome *c* (result not shown), supporting a previous suggestion (Ferguson-Miller *et al.*, 1976) that the effect of ATP is due to its binding to the oxidase rather than to cytochrome *c*.

The yield of labelling is about 0.1% (0.06% on subunit IV and 0.04% on subunit VIII). This low value is not unexpected in the light of the following consideration: (a) the yield of photo-induced cross-linking is highly variable, depending on the reactivities of the photo-generated intermediate and of the amino acid lateral

residues present in the binding site (Bisson & Montecucco, 1985); (b) our preliminary results indicate that the binding constant of ATP to cytochrome *c* oxidase is about 10^4 M^{-1} , and hence under the present conditions less than 5% of 8-azido-ATP is at any time bound to the oxidase; (c) a further factor affecting the yield of labelling as determined here is the position of the radioactive phosphorus atom, which can be lost after hydrolysis at the basic pH used during electrophoresis. For these reasons and because of the short life of ^{32}P , different photoactivatable ATP analogues will need to be used in further studies.

The present data are compatible both with the existence of a single binding site for ATP formed by the labelled subunits and with more sites located on different subunits. Although very unlikely, it cannot be excluded that other subunits are involved in binding ATP, but are not labelled because they do not contact the azido group or because they expose to it very unreactive amino acid residues (Bisson & Montecucco, 1985). What is shown here is that ATP does interact with cytochrome *c* oxidase, that its binding is specific and that it involves two

subunits coded for by nuclear genes: subunit IV and one of the subunits VIII.

Although it is established that the redox centres are localized on the three largest subunits, encoded on the mitochondrial DNA, no function has been so far demonstrated for the cytoplasmically synthesized subunits. However, several lines of evidence suggest that they may play a regulatory role on the enzyme *in vivo*, although these aspects are elusive for the present assays used *in vitro* (Kadenbach, 1983; Cumsky *et al.*, 1985).

The present paper suggests that a possible function of subunits IV and of one of the subunits VIII is to bind ATP. This binding appears to cause a long-range conformational change in the structure of the oxidase, resulting in a different ability of subunit II to bind cytochrome *c*. This idea is supported by independent evidence based on chemical modification and other labelling studies (C. Montecucco, G. Schiavo & R. Bisson, unpublished work).

The present approach should now allow us to identify by protein fragmentation and sequencing the precise sites of cross-linking and hence to map the ATP-binding site on cytochrome *c* oxidase in a way similar to that done by Hollemans *et al.* (1983) on the bovine mitochondrial F_1 -ATPase with a tritiated azido-ATP.

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