Anions induce conformational changes and influence the activity and photoaffinity-labelling by 8-azido-ATP of isolated cytochrome *c* oxidase

Achim REIMANN,* Fritz-Joachim HÜTHER,* Jan A. BERDEN† and Bernhard KADENBACH*

*Fachbereich Chemie, Biochemie, Philipps-Universität, Hans-Meerwein-Strasse, D-3550 Marburg, Federal Republic of Germany, and †B.C.P. Jansen Institute, Laboratory of Biochemistry, University of Amsterdam, 1000 HD Amsterdam, The Netherlands

The biphasic effect of anions on the activity of isolated bovine heart cytochrome *c* oxidase is paralleled by changes in the visible oxidized spectra, indicating the different conformational changes in the enzyme induced by bromide, chloride, sulphate, phosphate, ADP and ATP. Photoaffinity-labelling of most subunits of the isolated enzyme by low concentrations of 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is strongly increased by ATP, ADP and unlabelled 8-azido-ATP in an unspecific manner. With the reconstituted enzyme less subunits are labelled and this labelling is only little affected by nucleotides. The data suggest a highly dynamic structure for isolated bovine heart cytochrome *c* oxidase.

INTRODUCTION

Cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, contains a variable number of subunits depending on the evolutionary stage of the organism in which it is studied. The mammalian enzyme consists of 13 (Kadenbach *et al.*, 1983; Takamiya *et al.*, 1987), the yeast enzyme of 9 (Power *et al.*, 1984), and that from *Paracoccus denitrificans* of three subunits, which are homologous to the three mitochondrial-encoded subunits of the eukaryotic enzyme (Ludwig, 1987). In a recent hypothesis the 10 nuclear-encoded subunits of the mammalian enzyme were suggested to regulate the rate of respiration and the degree of coupling with proton translocation by binding allosteric effectors like substrates, ions, nucleotides, or hormones, resulting in conformational changes to the enzyme complex (Kadenbach, 1986). This hypothesis was supported by kinetic studies which demonstrated an increase in the K_m for cytochrome *c* of the reconstituted enzyme after photoaffinity labelling of soluble (Hüther & Kadenbach, 1986) or reconstituted (Hüther *et al.*, 1988) bovine heart cytochrome *c* oxidase with 8-azido-ATP. Furthermore, a decrease in the K_m for cytochrome *c* by intraliposomal ADP and an increase by intraliposomal ATP was seen with the bovine enzyme (Hüther & Kadenbach, 1987), but not with the enzyme from *P. denitrificans* (Hüther & Kadenbach, 1988).

The increase in the K_m for cytochrome *c* by 8-azido-ATP was prevented if ATP, but not ADP, was present during photolabelling (Hüther & Kadenbach, 1986; Hüther *et al.*, 1988). These kinetic results appear to have been corroborated by Montecucco *et al.* (1986), who found that specific labelling of subunits IV and VIII (VII) of soluble bovine heart cytochrome *c* oxidase by 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, was also protected by ATP, but not ADP, when present during photolabelling. In the present publication we found the opposite, namely a dramatic increase in photolabelling of the enzyme when ATP, ADP or unlabelled 8-azido-ATP was present. Explana-

tion for this unexpected finding is offered by the anion-induced conformational changes seen for isolated cytochrome *c* oxidase, which have a biphasic effect on enzyme activity (Kadenbach, 1986; Kadenbach *et al.*, 1987b, 1988) and change the visible spectra of the oxidized enzyme (Kadenbach *et al.*, 1988).

EXPERIMENTAL PROCEDURES

Enzyme preparation

Cytochrome *c* oxidase from bovine heart was prepared from isolated mitochondria as described before (Kadenbach *et al.*, 1986).

Preparation of proteoliposomes

The enzyme was reconstituted in liposomes by the cholates-dialysis method (Hüther & Kadenbach, 1986). *L*- α -Phosphatidylcholine [160 mg; type II-s from soybean (Sigma), purified by the method of Kagawa & Racker (1971)] was sonicated in 4 ml of 1.5% sodium cholate, 100 mM-K⁺/Hepes (pH 7.4) 50 mM-KCl to clarity. After addition of 12 nmol of cytochrome *aa*₃ the solution was dialysed twice for 4 h and once overnight against 100 mM-K⁺/Hepes (pH 7.4), 50 mM-KCl at 4 °C. The orientation of the enzyme was determined by the method of Casey *et al.* (1982).

Photoaffinity labelling

The isolated enzyme and proteoliposomes were photoaffinity labelled with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN Biochemicals, 40 Ci/mmol) in the buffer indicated in the legends to the Figures at a concentration of 6 μM - (soluble enzyme) or 3 μM - (proteoliposomes) haem *aa*₃. The reaction mixtures were preincubated in the dark for 15 min. Illumination was carried out in 200 μl of aliquots in a small dish (10 mm diameter) for 15–40 min at 0 °C with u.v. light (350 nm) at a distance of 4 cm from a CAMAG lamp (type TL 900, 8 W).

Fig. 1. Influence of increasing concentrations of anions on the activity of isolated bovine heart cytochrome *c* oxidase

The polarographic assay of activity was done in 1 mM-lauryl maltoside, 10 mM-K⁺/Hepes (pH 7.4), 7 mM-ascorbate, 0.7 mM-TMPD and 40 μ M-cytochrome *c* as described under Experimental procedures. The effectors were added as concentrated potassium solutions at pH 7.4. Each titration curve was recorded by continuous addition of the effector. (●), ATP; (■), ADP; (▲), KP_i; (○), KCl; (△), K⁺/Hepes.

SDS/polyacrylamide-gel electrophoresis

The enzyme in the labelled samples was precipitated by addition of 2 ml of 10 mM-NaH₂PO₄ and SDS/polyacrylamide-gel electrophoresis (PAGE) was performed as described by Kadenbach *et al.* (1983). Gels were stained with Coomassie Blue, destained, dried and autoradiographed, and the radioactive bands were cut out and counted in a liquid-scintillation counter as previously described (Hüther *et al.*, 1988). Lipids of labelled proteoliposomes were separated from protein by discontinuous density-gradient centrifugation (Zhang *et al.*, 1984). The protein pellet was washed with 10 mM-NaH₂PO₄.

Activity measurements

Cytochrome *c* oxidase activity was measured polarographically (Ferguson-Miller *et al.*, 1976) with a Gilson oxygraph at 25 °C in 10 mM-K⁺/Hepes (pH 7.4), 1 mM-lauryl maltoside, 25 mM-ascorbate, 40 μ M-cytochrome *c* (soluble enzyme), or in the indicated buffer containing 7 mM-ascorbate, 0.7 mM-*NNN'*-tetramethyl-1,4-

phenylenediamine dihydrochloride (TMPD), 0.1 mM-EDTA, and 0.02–40 μ M-cytochrome *c* (reconstituted enzyme), and the indicated amount of anion.

Measurements of spectra

Difference spectra of isolated oxidized cytochrome *c* oxidase at increasing concentrations of anions were recorded with a Uvikon 180 spectrophotometer (Figs. 4 and 5) or a Uvikon 860 spectrophotometer (Kontron) (Figs. 2 and 3) at 25 °C in 10 mM-K⁺/Hepes (pH 7.4), 1 mM-lauryl maltoside at a concentration of 4.9 μ M-haem *aa*₃. The indicated amounts of anions were added as concentrated potassium salt solutions (except Na₂SO₄). Stock solutions were 1 M (Cl⁻, Br⁻, SO₄²⁻, P_i) or 0.5 M (ADP, ATP). They were prepared in 10 mM-K⁺/Hepes, pH 7.4, and were adjusted to pH 7.4 with KOH.

RESULTS

In previous studies a biphasic effect of nucleotides and other anions on the activity of isolated cytochrome *c*

Fig. 2. Influence of increasing concentrations of ATP on the visible difference spectrum of oxidized cytochrome *c* oxidase

Both cuvettes contained an equal amount of isolated bovine heart cytochrome *c* oxidase ($4.9 \mu\text{M}$ -haem aa_3). The spectra were recorded immediately after addition of the indicated amount of ATP to the sample cuvette and an equal volume of the buffer to the reference cuvette. The time delay between the two spectra 6 min. For further details see under Experimental procedures.

oxidase from pig heart (Kadenbach, 1986) and rat liver (Kadenbach *et al.*, 1987b, 1988) was demonstrated, which at high anion concentrations results in almost complete inhibition of activity. Fig. 1(a) shows the influence of increasing concentrations of various anions on the activity of isolated bovine heart cytochrome *c* oxidase measured in the presence of TMPD and 7 mM-ascorbate. Except for the zwitterion Hepes, which stimulated enzyme activity up to a concentration of 30 mM and did not inhibit activity even above 200 mM, all anions stimulated activity at low concentrations and inhibited it at high concentrations. In general the effectiveness of anions appears to be related to ionic strength, but, as shown in Fig. 1(b), a plot of activity against ionic strength shows the specific effects of certain anions. Half-maximal inhibition with ADP was obtained at higher ionic strength than with chloride.

The effect of anions on enzyme activity is apparently due to conformational change in the isolated enzyme. This follows from their influence on the visible spectrum of the oxidized enzyme. In Fig. 2 the difference spectra for $4.9 \mu\text{M}$ -oxidized cytochrome *c* oxidase are recorded

after addition of increasing concentrations of ATP. The difference spectra are all characterized by maxima at 425, 560 and 603 nm and a shoulder at 445 nm. The absorbance differences at 445 and 603 nm, which are characteristic of the reduced enzyme, amount to 2–5 % of the absolute absorbance of the fully reduced enzyme at these wavelengths. The time delay in recording each spectrum was about 6 min. It was observed that upon prolonged incubation with the anion the spectrum changed slowly. Therefore, the time-dependent change in the difference spectrum induced by 25 mM-ATP was recorded (Fig. 3). Note that the presence of 25 mM-ATP reduced enzymic activity by about 50 % (Fig. 1). Whereas the maxima at 603 and 560 nm remained unchanged, that at 425 nm disappeared and two new maxima at 420 and 445 nm appeared within 60 min and did not change upon further incubation (results not shown). From these results we conclude that ATP induces two different conformational changes in soluble oxidized cytochrome *c* oxidase, a rapid and a slow one.

To record the rapid spectral change for various

Fig. 3. Time-dependent change in the visible difference spectrum of oxidized cytochrome *c* oxidase induced by 24 mM-ATP

Spectra were recorded at the indicated time after addition of ATP. For further details see under Experimental procedures.

concentrations of different anions we measured the spectra between 380 and 480 nm only (Fig. 4). The whole titration for each anion could be done in less than 17 min. Whereas the spectral changes for chloride and bromide were rather similar, all other spectra, including those for sulphate, phosphate, ADP and ATP, differed from each other. This result suggests that each anion induces specific conformational changes in soluble oxidized cytochrome *c* oxidase. The absorbance difference at 421–410 nm (Cl^- and Br^-), 424–410 nm (SO_4^{2-} and P_i) and 425–410 nm (ADP and ATP) and enzymic activity are plotted together against increasing anion concentrations in Fig. 5. In contrast with Fig. 1, the activity was measured in the absence of TMPD at 25 mM-ascorbate. A correlation between increase in absorbance difference and inhibition of enzyme activity was found for all anions at high concentrations.

Photoaffinity labelling of isolated (Hüther & Kadenbach, 1986) or reconstituted (Hüther *et al.*, 1988) cytochrome *c* oxidase with 8-azido-ATP increases the K_m for cytochrome *c*, which is protected by ATP but not by ADP, when present during illumination. Radioactive labelling of isolated cytochrome *c* oxidase by low concentrations of 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was strongly stimulated by addition of 10 μM - and even 100 μM -ATP (Fig. 6). In the presence of 10 mM-ATP, however, no

further increase in labelling was found. At a concentration of 100 μM , ADP stimulated labelling slightly less than ATP. Even more surprising was the effect of unlabelled 8-azido-ATP when added to the soluble enzyme during photolabelling. A further increase in labelling was seen with 1.1 μM and still further with 50 μM -8-azido-ATP, and was even stimulated further with 10 mM-ATP. These results, although performed under the same conditions, contradict those of Montecucco *et al.* (1986), who found protection, by ATP but not by ADP, against photoaffinity labelling of soluble bovine heart cytochrome *c* oxidase with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This can only be explained by assuming that anions induce strong conformational changes in the soluble enzyme which lead to accessibility of nascent binding sites for 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

Completely different results are obtained when reconstituted cytochrome *c* oxidase is photolabelled with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under similar conditions, except for the omission of laurylmaltoside and a prolonged illumination time (15 vs. 45 min) (Fig. 7). Only subunits II and/or III, IV and VIIa were labelled. ATP, ADP and unlabelled 8-azido-ATP had little influence on the intensity of labelling, except that some protection by 10 mM-ATP was found at 125 nM-8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Again an increase in labelling was found with unlabelled 8-azido-ATP, which was protected by ATP. This protection by ATP, however, was not specific, but was found equally for all labelled bands.

In further experiments we determined the total amount of 8-nitreno-ATP bound to each subunit at increasing concentrations of 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Fig. 8 shows the amount of nucleotide bound to isolated cytochrome *c* oxidase at increasing concentrations of 8-azido-ATP in the absence and presence of 10 mM-ATP or -ADP, given in mol of 8-nitreno-ATP bound per mol of subunit. Both nucleotides stimulated the photoaffinity labelling, but ATP to a greater extent. This result indicates that physiological nucleotide concentrations, i.e. 1–10 mM, apparently change the conformation of isolated cytochrome *c* oxidase, leading to improved binding or accessibility of binding sites for 8-azido-ATP and thus to increased photoaffinity labelling.

DISCUSSION

From recent publications it has become more and more evident that cytochrome *c* oxidase has a dynamic structure involving multiple conformational states. (1) During enzyme catalysis cytochrome *c* oxidase is suggested to change its conformational state. This was the conclusion of King *et al.* (1971) from o.r.d. and c.d. studies of the oxidized and reduced enzyme, and of Alleyne & Wilson (1987) from zinc cytochrome *c* fluorescence studies. Recently, Scholes & Malmström (1986) suggested that the enzyme underwent a transition from a 'closed' to an 'open' conformation upon reduction. (2) Kinetic and spectral data indicate different conformations for the resting and pulsed form of the isolated enzyme (Wilson *et al.*, 1981; Colosimo *et al.*, 1986); the extent of each form depends strongly on the experimental conditions (Antonini *et al.*, 1985). Complex conformational transitions between four different forms of the isolated enzyme (resting, pulsed, oxygenated and reduced) were suggested by Kornblatt & Luu (1986)

Fig. 4. Effect of increasing concentrations of various anions on the visible difference spectrum of isolated oxidized bovine heart cytochrome *c* oxidase

The enzyme was dissolved in 10 mM-K⁺/Hepes (pH 7.4), 1 mM-lauryl maltoside, at a concentration of 4.9 μ M-haem *aa*₃. The difference spectra between 380 and 480 nm were recorded immediately after addition of the indicated amount of anion to the sample cuvette and an equal volume of buffer to the reference cuvette. The time delay between the two spectra was 90 s. The position of the γ -band of reduced cytochrome *c* oxidase is indicated by the broken line, recorded at 40-fold lower scale expansion after addition of dithionite to the sample cuvette.

from fluorescence studies using porphyrin cytochrome *c*. From EXAFS data Naqui *et al.* (1984) suggested different active site structures for isolated enzyme preparations from different laboratories. (3) Regulation of cytochrome *c* oxidase activity by allosteric effectors has been suggested to involve conformational changes (Kadenbach, 1986). This hypothesis is supported by studies with the

reconstituted enzyme: intraliposomal phosphate changes the kinetics of cytochrome *c* oxidation of the bovine heart and liver enzyme to different extents (Büge & Kadenbach, 1986). ATP and ADP change the K_m for cytochrome *c* when acting from the cytosolic (Hüther & Kadenbach, 1986, 1988) or matrix side (Hüther & Kadenbach, 1987, 1988). The influence of ATP and

Fig. 5. Relationship between change in absorbance difference at selected wavelengths and enzyme activity at increasing concentrations of various anions

The absorbance differences at the indicated wavelengths for different concentrations of the anions were taken from the spectra in Fig. 5. The activity of cytochrome *c* oxidase was measured polarographically at increasing concentrations of the anions in 10 mM- K^+ /Hepes (pH 7.4), 1 mM-lauryl maltoside, 25 mM-ascorbate, 40 μ M-cytochrome *c* and 8.6 nM-cytochrome *c* oxidase.*

phosphate on the respiratory control ratio of the reconstituted bovine enzyme was described by Malatesta *et al.* (1987) and Kadenbach *et al.* (1988).

It was concluded from the changed oxidized spectra (Figs. 2–4) that conformational changes at the active site of isolated cytochrome *c* oxidase are induced by anions and can explain the inhibitory effect of high anion concentrations on enzyme activity (Figs. 1 and 5). Besides the general unspecific inhibition of activity, specific conformational changes in the enzyme induced by chloride or bromide, phosphate or sulphate, ADP and ATP are suggested by the different spectral changes. The identification of a rapid and a slow conformational change in the enzyme induced by ATP (Fig. 3) suggests multiple sub-states of the cytochrome *c* oxidase conformation similar to those described in detail for myoglobin (Ansari *et al.*, 1985). Strong conformational changes are also suggested by the stimulation of photoaffinity labelling with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by ATP, ADP and low concentrations of 8-azido-ATP, which is in contrast with their expected effect: a decreased incorporation of radioactivity. From Fig. 8 it may be

concluded that the positive co-operative binding of 8-azido-ATP only holds at low concentrations: in the mM range the binding of 8-azido-ATP was nearly proportional to the concentration. The protection against labelling by ATP reported by Montecucco *et al.* (1986) under identical conditions is not understood, but could be due to a different enzyme preparation. Results from Bisson *et al.* (1987) on the changed reactivity of carboxylic groups of isolated bovine heart cytochrome *c* oxidase, however, are in accord with a changed conformation of the binding domain for cytochrome *c*.

In summary, the present results support the view of a dynamic structure of cytochrome *c* oxidase, whose catalytic and structural properties are changed by multiple environmental factors, in particular by ions. The physiological interpretation of the results obtained with the solubilized enzyme may not be possible, because in the living cell the sidedness of ionic interaction would be essential for this transmembraneous enzyme complex.

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Fig. 6. Photoaffinity labelling of isolated bovine heart cytochrome *c* oxidase with 8-azido-[γ - 32 P]ATP

The isolated enzyme was photoaffinity labelled with 2×10^6 d.p.m. of 8-azido-[γ - 32 P]ATP (125 nM final concentration) in 25 mM-Tris/cacodylate buffer (pH 7.5), 0.05% lauryl maltoside for 15 min at 0 °C as described under Experimental procedures. Additions: lane 1, none; lane 2, 10 μ M-ATP; lane 3, 100 μ M-ATP; lane 4, 10 mM-ATP; lane 5, 100 μ M-ADP; lane 6, 1.1 μ M unlabelled 8-azido-ATP; lane 7, 50 μ M unlabelled 8-azido-ATP; lane 8, 50 μ M unlabelled 8-azido-ATP plus 10 mM-ATP. The enzyme was separated into subunits by SDS/PAGE as described under Experimental procedures.

Fig. 7. Photoaffinity labelling of reconstituted cytochrome *c* oxidase with 8-azido-[γ - 32 P]ATP

Proteoliposomes were prepared and photoaffinity labelled with 2×10^6 d.p.m. of 8-azido-[γ - 32 P]ATP (125 nM final concentration) in 100 mM- K^+ /Hepes (pH 7.5), 50 mM-KCl for 40 min at 0 °C with additions as described in the legend to Fig. 6. Removal of lipids and SDS/PAGE were performed as detailed under Experimental procedures.

Fig. 8. Total amount of 8-nitreno-ATP bound to individual subunits of isolated cytochrome *c* oxidase by photo-activation at increasing concentrations of radioactive 8-azido-ATP in the presence or absence of ATP or ADP

Cytochrome *c* oxidase (6 μ M) was photoaffinity labelled for 30 min with 8-azido-[γ - 32 P]ATP (2×10^6 d.p.m.) and increasing concentrations of unlabelled 8-azido-ATP (0, 0.2, 10, 100, 500, 750, 1000 and 5000 μ M) in the absence (○) or presence of 10 mM-ATP (□) or 10 mM-ADP (Δ). After SDS/PAGE and autoradiography, bands were cut out from the gel and counted as described under Experimental procedures. The question mark corresponds to an unknown radioactive band between subunits IV and Va (see Fig. 6).

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