Specific effects of ATP on the kinetics of reconstituted bovine heart cytochrome-*c* oxidase

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Bovine heart cytochrome-*c* oxidase was reconstituted in liposomes and the kinetics of cytochrome *c* oxidation were measured by the polarographic and photometric method under uncoupled conditions in the presence of various polyvalent anions. In order to distinguish between specific and unspecific ionic effects of ATP, the photolabelling reagent 8-azido-ATP was applied. Covalently bound ATP at the enzyme complex caused the same increase of K_m for cytochrome *c* as free ATP, if measured by the photometric assay. The increase of K_m by photolabelling with 8-azido-ATP was completely prevented by ATP, but not by ADP. The data indicate the occurrence of a specific binding site for ATP at the cytosolic side of cytochrome-*c* oxidase, which, after binding of ATP, changes the kinetics of cytochrome *c* oxidation.

Cytochrome-c oxidase *Photoaffinity labeling* *8-Azido-ATP* *Kinetics* *Nucleotide*

1. INTRODUCTION

The mammalian cytochrome-*c* oxidase complex is composed of 3 mitochondrial coded and 10 nuclear coded subunits [1]. The mitochondrial coded subunits contain the four redox centers and thus the catalytic activity. For the nuclear coded subunits a regulatory function was proposed [2,3]. It is generally known that the activity of isolated cytochrome-*c* oxidase is strongly dependent on the ionic strength and type of buffer [4,5], and on the method of assay [6]. Ferguson-Miller et al. [7] found a strong and noncompetitive inhibition of cytochrome *c* oxidation with Keilin-Hartree particles with the polyvalent anions ATP, ADP and P_i. In a recent paper, Montecucco et al. [8] described the specific binding of 8-azido-[γ-^{32}P]ATP to the nuclear coded subunits IV and VIII of isolated bovine heart cytochrome-*c* oxidase.

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-1,4-phenylenediamine dihydrochloride; 8-N_3-ATP, 8-azido-adenosine 5'-triphosphate

In this study we have investigated the effect of ATP on the activity of reconstituted bovine heart cytochrome-*c* oxidase and could distinguish between specific and unspecific effects by applying 8-N_3-ATP.

2. MATERIALS AND METHODS

L-α-Phosphatidylcholine, type II-s from soybean, and cytochrome *c*, type VI from horse heart, were obtained from Sigma, München. L-α-Phosphatidylcholine was purified by the method of Kagawa and Racker [9]. Valinomycin, CCCP and nucleotides were purchased from Boehringer, Mannheim and TMPD from Fluka, Neu-Ulm. 8-N_3-ATP was a gift of Dr H.-J. Schäfer, Mainz. Bovine heart cytochrome-*c* oxidase was prepared as described in [10] and reconstituted in liposomes by the cholate-dialysis method [11], with some modifications. The enzyme (6 nmol) dissolved in 1.5% Na cholate and the buffer indicated in the legends was mixed with 160 mg phosphatidylcholine, sonicated in 2 ml of 1.5% Na cholate and the indicated buffer, and dialyzed against three

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changes of the indicated buffer for 4 h (twice) and overnight. The orientation of cytochrome-*c* oxidase within the membrane was determined by the method of Casey et al. [12]. The respiratory control ratio of proteoliposomes was between 5 and 8.

Photolabelling of cytochrome-*c* oxidase with 8-N_3-ATP was performed according to Scheurich et al. [13]. The enzyme, dissolved in 20 μl of 0.7% Na cholate and the indicated buffer, was illuminated in a small dish (10 mm diameter) for 1 h at 0°C with ultraviolet light (350 nm) at 4 cm distance from a CAMAG type TL 900, 8 W lamp.

Initial rates of ferrocytochrome *c* oxidation by proteoliposomes were measured under uncoupled conditions by the polarographic and photometric assay, and presented in Eadie-Hofstee plots as described [14]. The activity is expressed as molecular turnover (TN = mole cytochrome *c*/s per mole cytochrome aa_3 at 25°C; *s* = concentration of cytochrome *c*).

3. RESULTS

The kinetics of cytochrome *c* oxidation of reconstituted cytochrome-*c* oxidase were measured polarographically under uncoupled conditions in the presence of various concentrations of polyvalent anions (fig.1). All investigated anions, e.g. ATP, ADP, pyrophosphate and phosphate, have a strong effect on the kinetics of cytochrome *c* oxidation by increasing the K_m of the high apparent K_m phase and decrease the proportion of the low apparent K_m phase as has been previously found with Keilin-Hartree particles by [7]. In

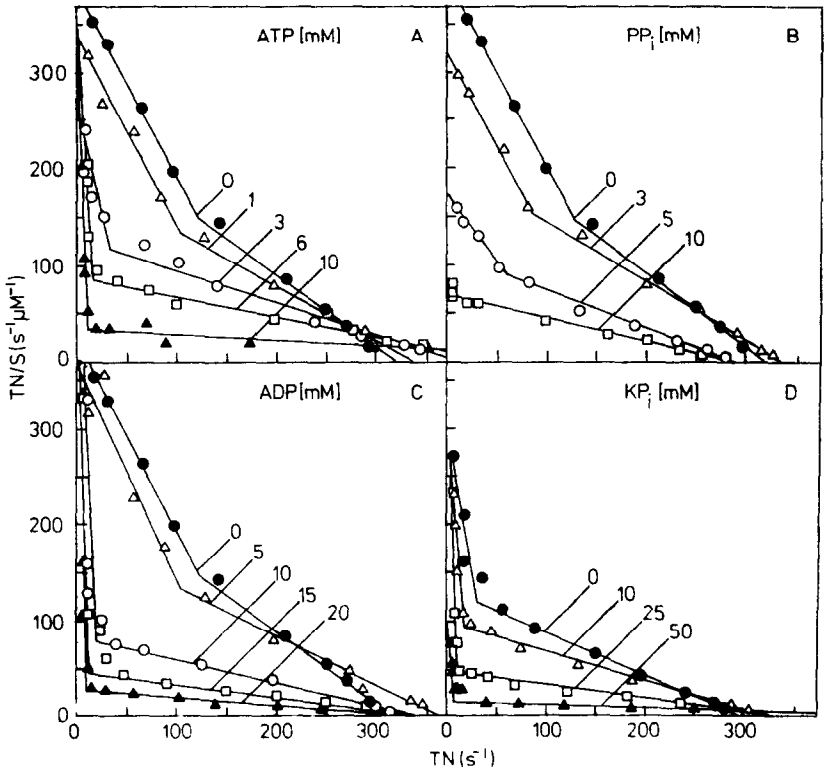

Fig.1. Concentration-dependent effects of ATP, ADP, and PP_i and P_i on the kinetics of cytochrome *c* oxidation by reconstituted bovine heart cytochrome-*c* oxidase. Cytochrome-*c* oxidase was reconstituted by dialysis against 50 mM KCl, 100 mM K Hepes, pH 7.2 (A, B, C), or against 25 mM KP_i, 50 mM KCl, 100 mM K Hepes, pH 7.2 (D). The activity was measured polarographically in 50 mM KCl, 100 mM K Hepes, pH 7.2, 1 μg/ml valinomycin, 3 μM CCCP, 0.7 mM TMPD, 25 mM K ascorbate, 0.02–40 μM ferricytochrome *c* and the indicated amount of anions. The orientation of the enzyme was 76% cytochrome *c*-binding site facing outwards in A, B and C and 85% in D.

fig.1D the intraliposomal buffer contained in addition 25 mM KP_i, which also affects the kinetics of cytochrome *c* oxidation by increasing the K_m for cytochrome *c* of the high apparent K_m phase. This result suggests a conformational change by intraliposomal phosphate of the binding domain for cytochrome *c* on the outside of proteoliposomes via the hydrophobic membrane domain of the enzyme complex as shown [15]. The similar effect of different polyvalent anions suggests an unspecific ionic effect. However, the effectiveness of anions was different and followed the order ATP > PP_i > ADP ≫ P_i.

The effect of ATP and phosphate on the kinetics of cytochrome *c* oxidation was also measured photometrically with proteoliposomes containing intraliposomal 100 mM KP_i, pH 6.0, as shown in fig.2. With 10 mM KP_i in the assay medium the lowest K_m was found (3.0 μM). Addition of 90 mM KP_i or 10 mM ATP increased the K_m to the same value (11.7 μM). Addition of 100 mM KP_i together with 10 mM ATP resulted in a further increase of the K_m (23.7 μM). Since a 9-fold concentration of phosphate is required to obtain the same effect as with ATP, but the valency of ATP is 4/3 that of phosphate, a specific effect of ATP cannot be excluded.

Montecucco et al. [8] described a specific photolabelling of subunits IV and VIII of soluble bovine heart cytochrome-*c* oxidase with 8-azido-[γ-^{32}P]ATP. We have photolabelled the soluble enzyme with 8-N_3-ATP and measured the kinetics of cytochrome *c* oxidation after reconstitution in liposomes by the polarographic method (fig.3). The 8-N_3-ATP-photolabelled and reconstituted enzyme has a higher K_m for the high apparent K_m phase of cytochrome *c* oxidation (fig.3A). This increased K_m was the same if photolabelling was done with 1, 5 or 10 mM 8-N_3-ATP (see inset in fig.3A), suggesting saturation of the ATP binding site(s) with 1 mM 8-N_3-ATP. Addition of 10 mM ATP to the photolabelled reconstituted enzyme increased the K_m to an even higher value (fig.3A), suggesting additional unspecific ionic effects of ATP on the steady-state oxidation of cytochrome *c*. Addition of 10 mM ATP to control proteoliposomes increased the K_m of the high K_m phase to the same value (not shown). Preincubation of the enzyme with 10 mM ATP prior to photolabelling with 8-N_3-ATP completely

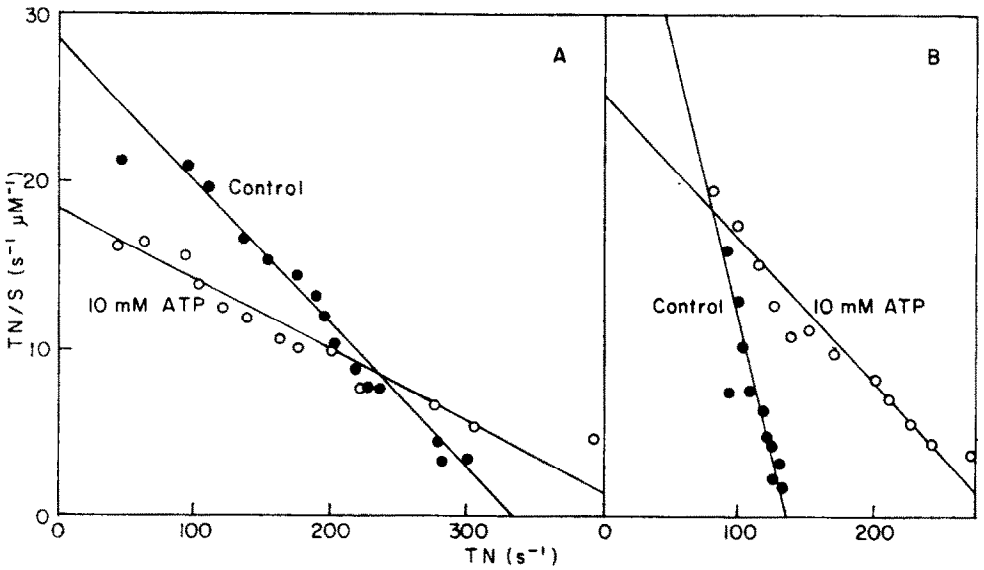

Fig.2. Effect of ATP on the kinetics of cytochrome *c* oxidation of reconstituted cytochrome-*c* oxidase measured photometrically at high and low phosphate concentrations. Cytochrome-*c* oxidase was reconstituted by dialysis against 100 mM KP_i, pH 6.0. The activity was measured photometrically in 100 mM KP_i, pH 6.0 (A), or 10 mM KP_i, pH 6.0, 150 mM sucrose (B) and 1 μg/ml valinomycin, 3 μM FCCP, 1–80 μM ferrocytochrome *c* and 10 mM ATP if indicated. The orientation of the enzyme was 75% cytochrome *c*-binding site facing outward in A and 77% in B.

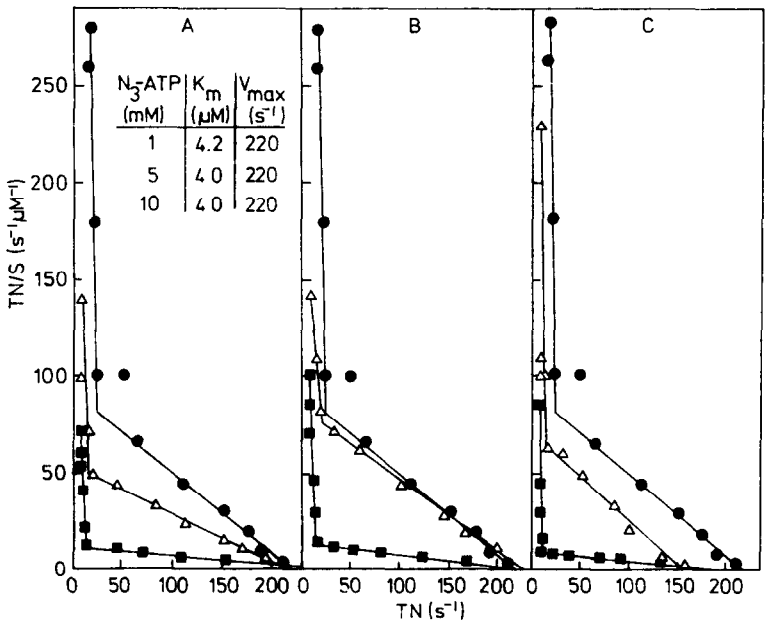

Fig.3. Modification by photolabelling with 8-N_3-ATP of the kinetics of cytochrome *c* oxidation of reconstituted cytochrome-*c* oxidase measured polarographically. 3 nmol cytochrome-*c* oxidase, dissolved in 200 μl of 0.7% Na cholate, 100 mM K Hepes, pH 7.2, 50 mM KCl, was preincubated for 15 min at 0°C with or without the indicated nucleotide. After addition of 0, 1, 5 or 10 mM 8-N_3-ATP (final concentration) photolabelling was performed as described in section 2. The enzyme samples were reconstituted by dialysis against 50 mM KCl, 100 mM K Hepes, pH 7.2, and the activity was measured polarographically as described in the legend to fig.1 with the indicated additions. The orientation of the enzyme was 85% cytochrome *c*-binding site facing outward for the control and for the 8-N_3-ATP-labelled enzyme, 77% for the ATP-protected 8-N_3-ATP-labelled enzyme, and 91% for the ADP-protected 8-N_3-ATP-labelled enzyme. (A) ●, control, preincubation with 10 mM ATP, illumination without 8-N_3-ATP, assay without ATP; △, preincubation without nucleotide, illumination with 8-N_3-ATP, assay without ATP; ■, preincubation without nucleotides, illumination with 8-N_3-ATP, assay with 10 mM ATP. (B) ●, control as in A; △, preincubation with 10 mM ATP, illumination with 8-N_3-ATP, assay without ATP; ■, preincubation with 10 mM ATP, illumination with 8-N_3-ATP, assay with 10 mM ATP. (C) ●, control as in A; △, preincubation with 10 mM ADP, illumination with 8-N_3-ATP, assay without ATP; ■, preincubation with 10 mM ADP, illumination with 8-N_3-ATP, assay with 10 mM ATP.

prevented the increase of K_m which, however, increased by addition of 10 mM ATP to the assay medium of the ATP-protected, 8-N_3-ATP-photolabelled reconstituted enzyme (fig.3B). Preincubation of the enzyme with 10 mM ADP decreased the V_{max} but scarcely affected the K_m (fig.3C). This result could indicate a photolabelling of the enzyme in the presence of ADP which, however, is different from that in its absence. Again 10 mM ATP in the medium of assay resulted in a large increase of K_m (fig.3C).

The kinetics of cytochrome *c* oxidation of the photolabelled reconstituted enzymes were also measured by the more physiological photometric method [21] as shown in fig.4. By this method the same increased K_m for cytochrome *c* is obtained, if the enzyme is either photolabelled with 8-N_3-ATP, or if 10 mM ATP is added to the unlabelled or photolabelled enzyme. Preincubation with 10 mM ATP (fig.4B), in contrast to ADP (fig.4C), prevented the increase of K_m after photolabelling. These results indicate specific binding of 8-N_3-ATP and of ATP to the cytochrome-*c* oxidase complex, accompanied by a decrease in affinity for cytochrome *c*.

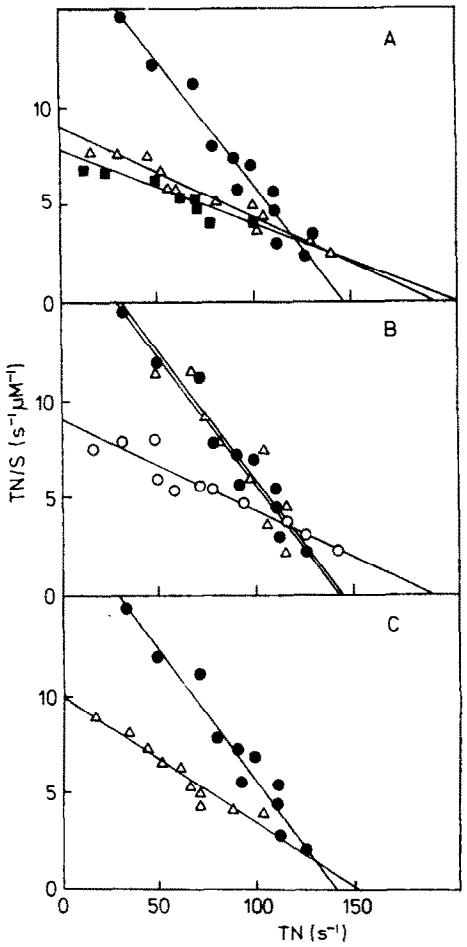

Fig.4. Modification by photolabelling with 8-N_3-ATP of the kinetics of cytochrome *c* oxidation of reconstituted cytochrome-*c* oxidase measured photometrically. Cytochrome-*c* oxidase was preincubated, photolabelled and dialyzed as described in the legend to fig.3. The activity was measured in 50 mM KCl, 100 mM K Hepes, pH 7.2, 1 μg/ml valinomycin, 3 μM CCCP, 1–80 μM ferrocytochrome *c* and the indicated nucleotide. The symbols are as described in the legend to fig.3, except for the additional symbol in B: ○, preincubation with 10 mM ATP, illumination without 8-N_3-ATP, assay with 10 mM ATP.

4. DISCUSSION

The inhibitory effects of ATP and ADP on the kinetics of cytochrome *c* oxidation by cytochrome-*c* oxidase were first described by Ferguson-Miller et al. [7] using Keilin-Hartree particles. The authors concluded that the inhibition resulted from anion interaction with the oxidase and suggested 'that this effect of ATP represents a possible mechanism for the control of electron flow to the oxidase'. In later studies, the inhibitory effects of polyvalent anions on cytochrome-*c* oxidase, however, were related to their binding to cytochrome *c* [5,16,17]. This view was based on the identification of specific binding sites for ATP and ADP [18], phosphate [18,20,21], citrate [16] and carbonate [19] on the cytochrome *c* molecule. But also monovalent anions were found to affect the kinetics of cytochrome *c* oxidation with Keilin-Hartree particles [7] or the isolated enzyme [3], as would be expected from the electrostatic nature of interaction between cytochrome *c* and cytochrome-*c* oxidase [22].

In order to distinguish between nonspecific ionic effects, or effects of anions due to binding to cytochrome *c*, and of specific interactions of allosteric effectors with the cytochrome-*c* oxidase complex, we have used the photoaffinity label 8-N_3-ATP which binds covalently to the enzyme in the absence of cytochrome *c* [8]. Covalently bound ATP increased the K_m for cytochrome *c* to the same extent as free ATP, if the activity of the reconstituted enzyme is measured by the photometric assay (fig.4). Only with the polarographic assay 10 mM ATP in the assay medium led to a further increase of the K_m. This could be explained by additional binding of ATP to cytochrome *c* [18] accompanied by decreased electron transfer from ascorbate to cytochrome *c* as measured in the polarographic assay. The protection by ATP of 8-N_3-ATP modification, but not by ADP (fig.4B and C), indicate a specific binding site for ATP on the cytochrome-*c* oxidase complex, which, upon binding of ATP, either covalently or noncovalently leads to a decreased affinity for cytochrome *c*. The binding site is suggested to be located at the cytosolic side of the enzyme complex, because free extraliposomal ATP results in the same change of the kinetics as covalently bound ATP (fig.4A and B). The different kinetics obtained after photolabelling with 8-N_3-ATP in the absence or presence of ADP, when assayed by the polarographic method (fig.3A and C), are not understood, but could be due to some interaction with an additional ADP binding

site at the complex (Hüther and Kadenbach, unpublished).

The data of this study support the view that there are three types of interactions of anions with the electron transfer system between cytochrome *c* and cytochrome-*c* oxidase: (i) the effect of monovalent anions which influence unspecifically the electrostatic interaction between cytochrome *c* and the complex [7]; (ii) the effect of certain polyvalent anions which bind to cytochrome *c* and influence the dissociation rate and/or redox potential of cytochrome *c* [5]; (iii) the specific effect of allosteric effectors like ATP which bind to cytochrome-*c* oxidase and modulate the kinetics of interaction with cytochrome *c* via conformational change as postulated [3].

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