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Influence of 8-Azido-ATP and Other Anions on the Activity of Cytochrome *c* Oxidase[1,2]

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Abstract

The effect of ATP and other anions on the kinetics of cytochrome *c* oxidation by reconstituted bovine heart cytochrome *c* oxidase was investigated. The following results were obtained: (1) ATP and other polyvalent anions increase the K_m for cytochrome *c* and the V_{max} (if assayed by the photometric method). The magnitude of the effect is proportional to the charge of the anion as follows from the series of increasing effectiveness: P_i < AMP < ADP < PP_i < ATP < PPP_i. (2) The kinetic effects are obtained in the millimolar physiological concentration range. (3) The kinetic changes are not saturated at high concentrations. (4) A specific interaction site for ATP at the cytosolic domain of the enzyme is concluded from the increase of K_m for cytochrome *c* after photolabelling of proteoliposomes with 8-azido-[γ-^{32}P]-ATP, which is protected by ATP but not by ADP. (5) No specific "binding site" for ATP could be identified by photolabelling with 8-azido-[γ-^{32}P]-ATP. The labelling is only partly protected by ATP or ADP.

Key Words: Cytochrome *c* oxidase; enzyme kinetics; photoaffinity labelling; nucleotides; proteoliposomes; regulation of activity.

Introduction

Cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, catalyzes the reduction of dioxygen to water with electrons from ferrocytochrome *c* and protons from the mitochondrial matrix, and in addition pumps protons. Its activity is a measure of the rate of cellular respiration. Since respiration is coupled with the synthesis of ATP via the

[1] Dedicated to Professor Dr. Friedhelm Schneider on the occasion of his 60th birthday.

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

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mechanism of oxidative phosphorylation, the activity of cytochrome *c* oxidase must be controlled by the energy state of the cell. According to the chemiosmotic hypothesis (Mitchell, 1961), the rate of mitochondrial electron transport is controlled by the proton electrochemical potential $\Delta\tilde{\mu}_{H^+}$ across the inner mitochondrial membrane. A number of more recent observations has been interpreted as being inconsistent with the view that $\Delta\tilde{\mu}_{H^+}$ is the only rate-controlling parameter, and with a delocalized model of free energy coupling [for reviews see Westerhoff *et al.* (1984), Ferguson (1985), and Rottenberg (1985)]. Instead direct interactions between redox and ATPase pumps and/or a proton flow within or adjacent to the membrane have been proposed for free energy coupling of proton pumps (Rottenberg, 1978; Westerhoff *et al.*, 1984; Slater *et al.*, 1985). In addition allosteric effectors (metabolites, cofactors, ions, and hormones) have been proposed to influence the rate of respiration by direct interaction with cytochrome *c* oxidase (Kadenbach, 1986).

This hypothesis was based on the occurrence of 10 different nucleus-encoded subunits in addition to the three catalytic mitochrondria-encoded subunits of mammalian cytochrome *c* oxidase (Kadenbach *et al.*, 1983). Some of the nucleus-encoded subunits occur in multiple tissue- and developmental-specific forms (Kuhn-Nentwig and Kadenbach, 1985), and were assumed to modify the kinetic properties of the enzyme (Büge and Kadenbach, 1986).

In two previous studies the effect of ATP on the oxidation of ferrocytochrome *c* by cytochrome *c* oxidase from bovine heart (Ferguson-Miller *et al.*, 1976) and yeast (Roberts and Hess, 1977) was studied. These studies, however, could not distinguish between interaction of ATP with the oxidase or with cytochrome *c*, which is known to bind specifically ATP (Corthesy and Wallace, 1986). By using the photoaffinity probe 8-azido-ATP, we could demonstrate specific changes of the kinetics of cytochrome *c* oxidation after covalent binding to cytochrome *c* oxidase (Hüther and Kadenbach, 1986).

In the present study 8-azido-[γ-^{32}P]-ATP was applied in order to identify the site of interaction in reconstituted cytochrome *c* oxidase. In addition the effect of other polyvalent anions on the kinetics of cytochrome *c* oxidation was measured. The data suggest complex interactions of nucleotides and other anions with the redox couple cytochrome *c*/cytochrome *c* oxidase.

Materials and Methods

Sodium tripolyphosphate was purchased from Janssen Chimica, Nettetal. 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

(1971). Valinomycin, CCCP, and nucleotides were purchased from Boehringer, Mannheim, and TMPD from Fluka, Neu-Ulm. 8-Azido-adenosine-5′-triphosphate, γ-^{32}P triethylammonium salt (10–40 Ci/mmol), was either bought from ICN Biomedicals, or was synthesized as described previously (Heaton *et al.*, 1978). Unlabelled 8-N_3-ATP was synthesized as described in (Schäfer *et al.*, 1978).

Cytochrome *c* oxidase was isolated from bovine heart mitochondria (Kadenbach *et al.*, 1986) and reconstituted in liposomes by the cholate-dialysis method. Phosphatidylcholine (160 mg) was sonicated in 4 ml 1.5% sodium cholate, 100 mM K-Hepes, pH 7.4, and 50 mM KCl to clarity. After addition of 12 nmol cytochrome aa_3, corresponding to a lipid/protein ratio 67/ (w/w), the solution was dialyzed twice for 4 h and once overnight against 100 mM K-Hepes, pH 7.4 and 50 mM KCl at 4°C. The orientation of cytochrome *c* oxidase within the membrane was 75–86% right-side out, as determined by the method of Casey *et al.* (1982). Proteoliposomes (200 μl) were photoaffinity-labelled with 8-N_3-[γ-^{32}P]-ATP in a small dish (10 mm diameter) for 40 min at 0°C with ultraviolet light (350 nm) at 4 cm distance from a CAMAG type TL 900, 8-W lamp. Kinetic measurements were performed after removal of unbound nucleotide by dialysis.

Photoaffinity-labelling of proteoliposomes with 8-N_3-[γ-^{32}P]-ATP was carried out as described above with additions indicated in the legends to the figures. The lipids were removed from the enzyme by discontinuous density gradient centrifugation as described by Zhang *et al.* (1984). The protein pellet was washed with 10 mM NaH_2PO_4, and SDS-gel electrophoresis was performed as described (Kadenbach *et al.*, 1983). After staining, the gels were dried and autoradiographed, and radioactive bands were cut out, dissolved in 1 ml H_2O_2, and counted in a liquid scintillation counter.

Cytochrome *c* oxidase activity of proteoliposomes was measured by the polarographic assay in 100 mM K-Hepes, pH 7.4, 50 mM KCl, 25 mM K-ascorbate, 0.7 mM TMPD, 0.1 mM EDTA, 0.02–40 μM cytochrome *c*, in the presence or absence of 1 μg/ml valinomycin, and 3 μM CCCP as indicated. Initial rates of ferrocytochrome *c* oxidation were determined by the photometric assay in 100 mM K-Hepes, pH 7.4, 50 mM KCl, 1–80 μM ferrocytochrome *c*, in the presence and absence of 1 μg/ml valinomycin, and 3 μM CCCP. The kinetic data were determined from Eadie–Hofstee plots as previously described (Büge and Kadenbach, 1986).

Results

In Fig. 1 is presented the effect of externally added adenine nucleotides on the K_m and V_{max} for cytochrome *c* of reconstituted cytochrome *c* oxidase,

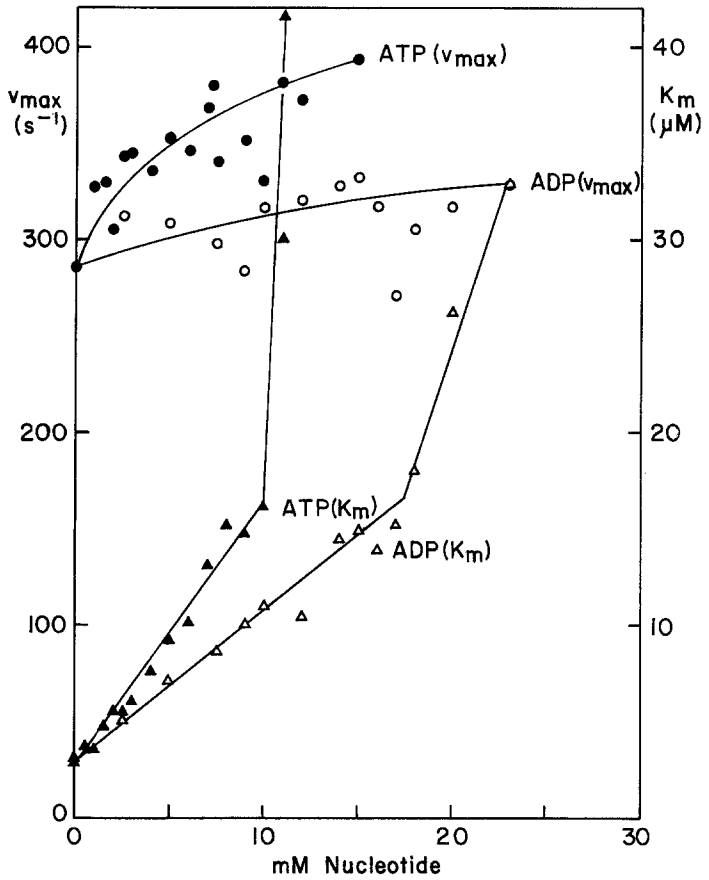

Fig. 1. Influence of ATP and ADP on the kinetic parameters of reconstituted bovine heart cytochrome *c* oxidase. Cytochrome *c* oxidase activity in proteoliposomes was measured at various cytochrome *c* concentrations in the presence of uncouplers by the polarographic assay. The K_m's for cytochrome *c* and the V_{max} values were calculated from Eadie–Hofstee plots from the low-affinity phase of reaction. Closed symbols: ATP; open symbols: ADP; circles: V_{max} values; triangles: K_m values.

as determined by the polarographic assay. Each value was calculated from individual Eadie–Hofstee plots for the low-affinity phase of cytochrome *c* oxidation (Ferguson-Miller *et al.*, 1976). ATP and ADP affect the V_{max} of cytochrome *c* oxidation only to a small extent, but strongly increase the K_m for cytochrome *c*. With increasing nucleotide concentrations no saturation is obtained, but instead an abrupt further increase of K_m is seen at 10 mM ATP and 18 mM ADP, which, however, was not found when measured by the photometric assay.

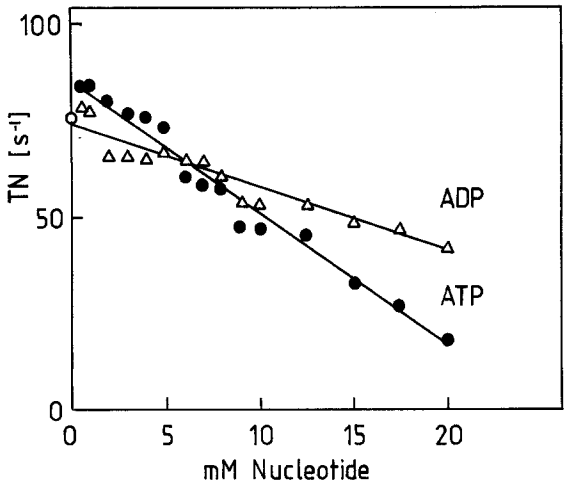

Fig. 2. Influence of increasing concentrations of ATP and ADP on the activity of reconstituted cytochrome *c* oxidase determined by the photometric assay. Cytochrome *c* oxidase activity was measured under coupled conditions in the presence of 6 μM ferrocytochrome *c* at the indicated concentrations of nucleotides. The data are averages from two determinations. Closed symbols: ATP; open symbols: ADP.

The effect of increasing nucleotide concentrations on the photometrically determined activity of reconstituted cytochrome *c* oxidase at 6 μM ferrocytochrome *c* concentration is presented in Fig. 2. From this presentation the following results are obtained: (a) The data for ATP and ADP are on straight lines, indicating no saturation of the nucleotide effect as found with the polarographic method. (b) The data with ATP and ADP are on different straight lines, indicating different efficiencies of inhibition for the two nucleotides. Nucleotides stimulate the V_{max} of cytochrome *c* oxidation when measured by the photometric assay as compared to the polarographic assay (Table I). Therefore the decrease of activity at 6 μM cytochrome *c* caused by the nucleotides (Fig. 2) reflects an increase of the K_m for cytochrome *c*, as is also found with the polarographic method (Fig. 1).

In order to explore the nature of nucleotide interaction with the enzyme, the effect of other anions on the K_m and V_{max} of reconstituted cytochrome *c* oxidase was studied (Table 1, A and B). Pyrophosphate and tripolyphosphate also stimulate the K_m for cytochrome *c*, the latter even to a larger extent than ATP. Clearly the effect of anions is proportional to the valence of the anion. The following order of effectiveness is found: P_i < AMP < ADP < PP_i < ATP < PPP_i, suggesting that the charge of the anion contributes rather to the increase of K_m for cytochrome *c* than the adenosine moiety of nucleotides.

Table I. Influence of Various Anions on the Kinetic Parameters of Reconstituted Bovine Heart Cytochrome *c* Oxidase[a]

	Additions	Polarographic assay		Photometric assay	
		K_m (μM)	V_{max} (s^{-1})	K_m (μM)	V_{max} (s^{-1})
(A)	5 mM				
	None	1.1	280	15.8	310
	IDP	1.3	290		
	ADP	1.1	295		
	PP_i			18.4	350
	ITP	3.4	280	26.6	420
	ATP	3.3	310	33.3	470
(B)	10 mM				
	None	2.7	300	10.4	150
	Cl^-	2.6	314		
	Acetate	3.6	280		
	P_i	5.2	310		
	AMP	5.6	308		
	ADP	8.3	315		
	PP_i	11.6	306		
	ATP	13.0	312	27.1	190
	ITP	13.6	315		
	PPP_i	18.5	305	41.2	210
(C)	10 mM				
	None + valinomycin/CCCP			10.5	136
	None − valinomycin/CCCP			12	31
	ATP − valinomycin/CCCP			29	38
(D)	10 mM				
	None			10.4	150
	8-N_3-adenosine			10.0	135
	8-N_3-ATP	21.8	180		

[a] All data were measured with proteoliposomes in the presence of valinomycin and CCCP, except in (C) as indicated. Proteoliposomes in (A) and (B), determined photometrically, were prepared with different enzyme preparations. The polarographic and photometric assay was performed as described in Materials and Methods, and the kinetic data were calculated from the low-affinity phase of cytochrome *c* oxidation. The kinetic measurements in (D) were carried out after photolabelling of proteoliposomes with the indicated compounds as described in Materials and Methods.

As found previously (Hüther and Kadenbach, 1986), the effects on the K_m are more pronounced when assayed polarographically, but with the photometric assay the V_{max} is more strongly stimulated.

In Table I, C the effect of ATP on cytochrome *c* oxidase in coupled proteoliposomes is shown. The respiratory control ratio of the proteoliposomes is 4.5, but the K_m for cytochrome *c* is almost the same when measured photometrically in the absence or presence of uncouplers. Also the increase of K_m by ATP is the same under coupled (Table I, C) and uncoupled (Table I, B) conditions.

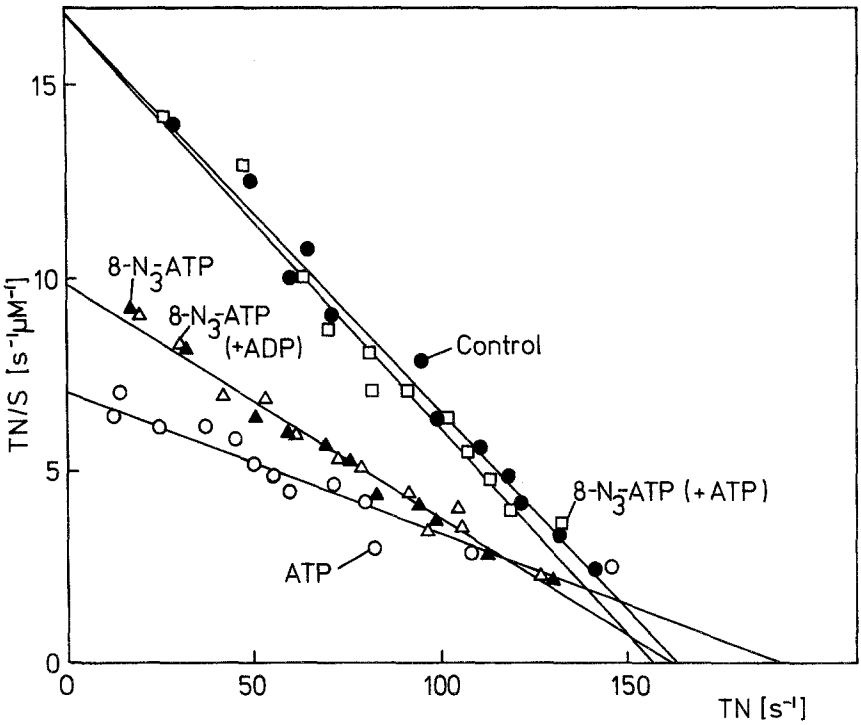

Fig. 3. Change of the K_m for cytochrome *c* of proteoliposomes after photolabelling with 8-N_3-ATP as measured by the photometric method. The photolabelling and the photometric assay of activity of proteoliposomes at various cytochrome *c* concentrations was carried out as detailed under Methods. (●) Control, illumination of proteoliposomes without additions; (○) illumination without additions, assay in the presence of 20 mM ATP; (▲) photolabelling with 10 mM 8-N_3-ATP; (□) photolabelling with 10 mM 8-N_3-ATP in the presence of 10 mM ATP; (△) photolabelling with 10 mM 8-N_3-ATP in the presence of 10 mM ADP.

Photoaffinity-labelling of proteoliposomes, prior to the assay, with 10 mM 8-N_3-ATP (Table I, D) increases the K_m for cytochrome *c* to 210%, whereas 10 mM free ATP, added to the proteoliposomes during the photometric assay, increases the K_m value to 260%. Photolabelling with 10 mM 8-N_3-adenosine does not affect the kinetic properties of the proteoliposomes. These results indicate that the effect of ATP on the kinetics of cytochrome *c* oxidation is mainly due to electrostatic interaction with the cytochrome *c* oxidase and not with cytochrome *c*.

The protection by ATP but not by ADP of the increase of K_m for cytochrome *c* upon photolabelling of the reconstituted enzyme with 8-N_3-ATP is shown in Fig. 3. ATP (10 mM) but not ADP (10 mM) present during photolabelling with 8-N_3-ATP, completely prevents the increase of the K_m. From Table II it follows that concentrations of 8-N_3-ATP above 3 mM but

Table II. Effect of Photolabelling with 8-N_3-ATP of Reconstituted Cytochrome *c* oxidase on the Photometrically Measured Kinetics of Cytochrome *c* Oxidation[a]

8-N_3-ATP (mM)	Nucleotide (mM)		K_m (μM)	V_{max} (s^{-1})
	Added before photolabelling	Added in assay		
0	—	—	9.9	165
0	—	10 ATP	26.4	190
1	—	—	8.8	150
3	—	—	11.4	160
10	—	—	16.0	160
10	—	10 ATP	26.4	185
10	10 ATP	—	8.8	155
10	10 ADP	—	16.0	160

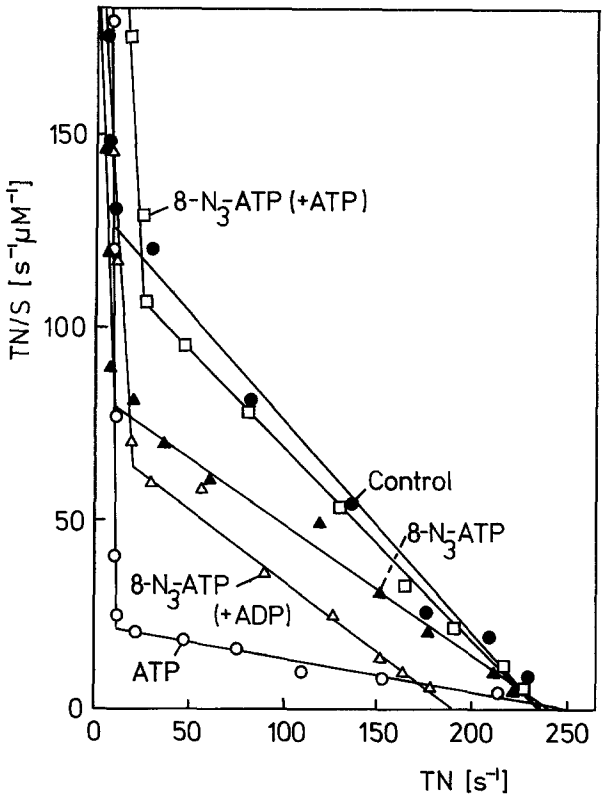

Fig. 4. Change of the K_m for cytochrome *c* of proteoliposomes after photolabelling with 8-N_3-ATP as measured by the polarographic method. Photolabelling and the polarographic assay of activity of proteoliposomes at various cytochrome *c* concentrations was carried out as described in Materials and Methods.

not below 1 mM are effective, indicating a low affinity of 8-N_3-ATP for the site involved in the regulatory effect.

The data of Fig. 3 were measured by the photometric assay. Similar effects were found when the kinetic data were measured by the polarographic assay as shown in Fig. 4. Again the presence of 10 mM ATP almost completely protected the increase of K_m by photolabelling with 8-N_3-ATP. The presence of 10 mM ADP had almost no protective effect, but it decreased the V_{max} of the reconstituted enzyme when present during photolabelling with 8-N_3-ATP. This result was already found previously by photolabelling of the soluble enzyme (Hüther and Kadenbach, 1986) and indicates an additional specific effect of ADP.

The binding of 8-N_3-ATP to reconstituted cytochrome *c* oxidase was studied by using the [γ-^{32}P]-labelled compound. In Fig. 5 the Coomassie blue staining pattern and the autoradiography of the labelled and gel-electrophoretically separated enzyme complex are shown. Photolabelling for 10 min (lane 2) instead of 40 min (lanes 3–8) is not sufficient for a measurable level of covalent attachement of the azido compound. Photolabelling for

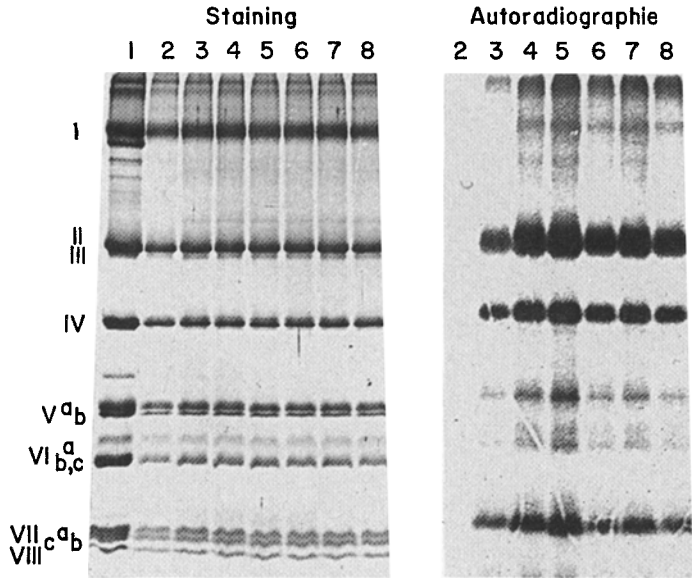

Fig. 5. SDS gel electrophoresis of cytochrome *c* oxidase photolabelled with 8-N_3-[γ-^{32}P]-ATP after reconstitution in liposomes. Proteoliposomes were photolabelled for 10 min (lane 2) or 40 min (lanes 3–8) with the indicated additions. The concentration of 8-N_3-[γ-^{32}P]-ATP was 20 μM (lanes 2 and 3), 50 μM (lanes 4, 6–8), and 100 μM (lane 5). Before photolabelling, 1 mM ATP (lane 6), 10 mM ATP (lane 7), or 10 mM ADP (lane 8) were added. For further details see Materials and Methods.

more than 40 min, however, does not further increase the bound radioactivity (not shown). With increasing concentrations of 8-N_3-[γ-^{32}P]-ATP from 20 to 100 μM, the bound radioactivity increases (lanes 3–5), but no specific labelling is found. Radioactivity is found in subunits II (and or III), IV, Va/b, VIa, VIb/c, and VIIa/b/c. Subunits II, IV, and VIIa/b/c are most intensively labelled. The presence of 1 or 10 mM ATP or 10 mM ADP (lanes 6–8,

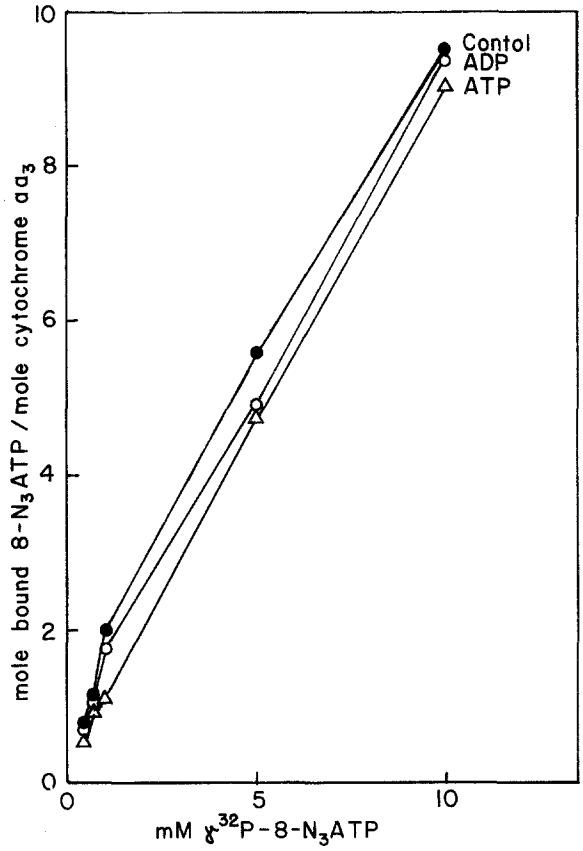

Fig. 6. Concentration dependence of photolabelling of proteoliposomes with 8-N_3-[γ-^{32}P]-ATP. 100 μl of reconstituted cytochrome *c* oxidase each was photolabelled with 0.07 μCi 8-N_3-[γ-^{32}P]-ATP diluted with unlabelled 8-N_3-ATP to the indicated final concentration (●) in the presence of 10 mM ATP (△) or ADP (○) as described in Materials and Methods. The unbound radioactive nucleotide was separated by gel chromatography (Sephacel G-75, 5 cm in pasteur pipettes) and the radioactivity was counted in a liquid scintillation counter according to Cerenkov. The unspecific labelling of phospholipids by 8-N_3-[γ-^{32}P]-ATP was measured under identical conditions with enzyme-free liposomes at 0.04 μM and 10 mM radioactive nucleotide. The radioactivity bound to enzyme-free liposomes amounted to 41 ± 9% at 0.04 μM and to 20 ± 13% at 10 mM 8-N_3-[γ-^{32}P]-ATP, as compared to the radioactivity bound to proteoliposomes (average of four individual determinations).

respectively) during photolabelling has no marked influence either on total or specific labelling of subunits. This contrasts with the complete protective effect of 10 mM ATP on the increase of K_m for cytochrome *c* by photolabelling with 8-N_3-ATP (Fig. 3).

The covalent binding of 8-N_3-[γ-^{32}P]-ATP to proteoliposomes shows a linear increase up to 10 mM (Fig. 6). This binding is affected by the presence during photolabelling of 10 mM ATP or, to a lesser extent, 10 mM ADP. At 2, 5, and 10 mM 8-N_3-[γ-^{32}P]-ATP about 0.5–1 mole ATP-sensitive labelling is found. The data of Fig. 6 have to be corrected for an unspecific covalent binding to phospholipids. At 0.04 μM and 10 mM 8-N_3-[γ-^{32}P]-ATP 41 and 20% of the radioactivity indicated in Fig. 6 was found bound to phospholipids, respectively (average of four determinations), as determined by photolabelling of protein-free liposomes.

Discussion

The present paper extends the observation of a preceding publication, where an increase of the K_m for cytochrome *c* of reconstituted cytochrome *c* oxidase was demonstrated (Hüther and Kadenbach, 1986) by soluble ATP as well as by covalently bound 8-nitreno-ATP, in several aspects:

1. In addition to ATP, other anions with high negative charge density (e.g., polyvalent anions) have a similar or even stronger effect on the K_m for cytochrome *c*, as found, e.g., with the tripolyphosphate anion. The effect is proportional to the number of charges of the anion. The following series of anions of increasing effectiveness was obtained: P_i < AMP < ADP < PP_i < ATP < PPP_i. Covalently bound 8-nitreno-adenosine, in contrast to 8-nitreno-ATP, has no effect on the kinetic properties of the enzyme.

2. The increase of the K_m for cytochrome *c* by polyvalent anions does not show a saturation behavior. The interaction does not occur at low micromolar concentrations, but in the millimolar "physiological" concentration range.

3. The interaction of ATP with cytochrome *c* oxidase occurs on the cytosolic side of the enzyme complex. This follows from the similar effect of covalently bound ATP and of free ATP on the reconstituted enzyme. Photolabelling with 8-N_3-ATP in this study was performed with proteoliposomes, whereas in the preceding publication the isolated enzyme was photolabelled and the activity determined after reconstitution (Hüther and Kadenbach, 1986).

4. No specific binding of 8-N_3-[γ-^{32}P]-ATP to subunits IV or VII (VIII) of the enzyme was found. This observation contrasts with the data of

Montecucco *et al.* (1986), who report specific binding to these subunits upon photolabelling of the soluble enzyme with 8-Ni_3-[γ-^{32}P]-ATP. However, a specific binding site for ATP cannot be excluded to occur at the cytosolic side of cytochrome *c* oxidase. This follows from the protection by ATP of 0.5–1 mole bound 8-nitreno-[γ-^{32}P]-ATP per mole cytochrome aa_3, when present during photoactivation (Fig. 6) (see also Bisson *et al.*, 1987).

5. A specific binding site for ATP is also concluded from the protection by ATP but not by ADP of the increase of K_m for cytochrome *c* by photolabelling of proteoliposomes with 8-N_3-ATP (Fig. 2), as has been found with the soluble enzyme (Hüther and Kadenbach, 1986).

The apparent contradiction between the unspecific effect of anions on the increase of K_m for cytochrome *c*, depending only on the valence of the anion (Table I), and a specific protection by ATP but not by ADP of the increase of K_m for cytochrome *c* during photolabelling with 8-N_3-ATP (Figs. 3 and 4), could be due to different types of interactions of anions and nucleotides with the cytochrome *c*/cytochrome *c* oxidase redox system. In fact, three different types of interactions can be distinguished: (i) Specific binding sites for phosphate (Ferguson-Miller *et al.*, 1978), bicarbonate (Osheroff *et al.*, 1980), citrate (Osheroff *et al.*, 1978), and nucleotides (Stellwagen and Shulman, 1973; Corthesy and Wallace, 1986) have been identified at the basic cytochrome *c* molecule, and these have been suggested to influence the kinetics of ferrocytochrome *c* oxidation (Osheroff *et al.*, 1978). (ii) The electrostatic interaction between cytochrome *c* and the oxidase is expected to be influenced by the ionic strength of the medium (Koppenol and Margoliash, 1982). (iii) A specific binding of ATP to the cytosolic domain of cytochrome *c* oxidase is concluded from Fig. 3 and 4, but in this latter case the kinetic changes can be deduced to binding of 8-nitreno-ATP to the oxidase. In all other experiments, where the effect of soluble anions on the kinetics of cytochrome *c* oxidation was investigated, no decision between the three possible interactions can be made.

The lack of saturation of the increase of K_m for cytochrome *c* at high anion concentrations (Figs. 1 and 2) could be due to the unspecific effect of the ionic strength of the medium, which might become particularly effective at high concentrations.

The steep increase of K_m for cytochrome *c* at very high nucleotide concentrations, as found with the polarographic (Fig. 1) but not with the photometric method of assay (Fig. 2), could be due to ionic interaction (competition) with ascorbate and/or TMPD, which are not included in the photometric assay.

The failure to detect a specific labelling of a subunit of cytochrome *c* oxidase by 8-N_3-[γ-^{32}P]-ATP could partially be due to its unnatural

configuration as compared to ATP (Berden *et al.*, 1985). In future work, labelled 2-N_3-ATP, whose structure more closely resembles that of ATP, will be applied in order to identify the possibly specific binding site for ATP at the cytoplasmic domain of the enzyme.

It should be pointed out that other binding sites for nucleotides have been postulated to occur at the matrix domain of cytochrome *c* oxidase by kinetic studies: one for ADP, which decreases the K_m for cytochrome *c*, and another for ATP, which increases the K_m for cytochrome *c* (Hüther and Kadenbach, 1987). Thus a detailed description of the "physiological" interaction of nucleotides with cytochrome *c* oxidase awaits further investigations. Recently a regulatory effect of external ATP on cytochrome *c* oxidase was also concluded by Rigoulet *et al.* (1987) from spectrometric measurements with yeast mitochondria.

Acknowledgments

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