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THE NUMBER OF NUCLEOTIDE BINDING SITES IN CYTOCHROME C OXIDASE

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The binding of 2'(3')-O-(2,4,6-trinitrophenyl)-adenosine-5'-triphosphate (TNP-ATP), [^{35}S]ATPαS and 8-azido-[γ-^{32}P]ATP to isolated cytochrome c oxidase of bovine heart and liver and to the two-subunit enzyme of *Paracoccus denitrificans* was studied by measuring the fluorescence change or bound radioactivity, respectively. With TNP-ATP three binding sites were determined at cytochrome c oxidase from bovine heart and liver, both with two dissociation constants K_d of about 0.2 and 0.9 μM. Trypsin treatment of the enzyme from bovine heart, resulted in one binding site with a K_d of 0.3 μM. The two-subunit enzyme of *Paracoccus denitrificans* had only one binding site with a K_d of 3.6 μM.

The binding of [^{35}S]ATPαS to cytochrome c oxidase was studied by equilibrium dialysis. With the enzyme of bovine heart seven and the enzyme of liver six high-affinity binding sites with apparent K_d's of 7.5 and 12 μM, respectively, were obtained. The two-subunit enzyme of *Paracoccus denitrificans* had one binding site with a K_d of 20 μM. The large number of binding sites at cytochrome c oxidase from bovine heart, mainly at nuclear coded subunits, was verified by photoaffinity labelling with 8-azido-[γ-^{32}P]ATP.

Cytochrome c oxidase (COX), the terminal enzyme of some bacterial and the mitochondrial respiratory chain, catalyses the reduction of molecular oxygen to water, coupled with the storage of energy in an electrochemical proton gradient across the membrane. The "proton motive force" is used by the ATP synthase for the generation of ATP from ADP and phosphate. Recently the X-ray crystal structure of COX from *Paracoccus denitrificans* [1] and from bovine heart [2] at 2.8 Å resolution have been published and verified various structural features, previously ascertained by biochemical methods. These include four protein subunits in COX of *Paracoccus* [3], and 13 subunits in COX of bovine heart [4]. In eucaryotes subunits I-III are encoded on mitochondrial, the remaining subunits on nuclear DNA. Isozymes of COX were identified, based in mammals on tissue-specific expression of two isoforms of subunits VIa, VIIa and VIII [5,6].

Nuclear coded subunits were suggested to bind allosteric effectors and to regulate the activity of COX [7]. The interactions of ATP and ADP with COX have been studied for almost 20 years. Adenine nucleotides result in changed kinetics [8-15], and modified visible spectra [16-19].

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Abbreviations: TNP-ATP, 2'(3')-O-(2,4,6-Trinitrophenyl)-adenosine-triphosphate, COX, cytochrome c oxidase.

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Nucleotides interact with nuclear coded COX subunits, as concluded from opposite effects of intraliposomal ADP and ATP on the K_m for cytochrome c of reconstituted COX from bovine heart but not from *Paracoccus* [13]. Intraliposomal nucleotides were also shown to act tissue-specifically with COX from bovine heart, but not from bovine liver [20], and to influence the rate of respiration as well as efficiency of energy transduction, depending on the intraliposomal ATP/ADP ratio [21,22]. High intraliposomal ATP/ADP ratios decrease the H^+/e^--stoichiometry of reconstituted COX from bovine heart (Frank and Kadenbach, in preparation). With the photoaffinity reagents 8-azido-[γ-^{32}P]ATP [23] and [γ-^{32}P]ATP [15] two subunits (IV and VII) of isolated COX from bovine heart were labelled. In contrast, labelling of multiple subunits (II, IV, Vab, VIa, VIbc and VIIabc) was obtained in other studies under the same conditions and assumed to represent unspecific labelling [13,17]. In contrast, with 2-azido-[β,γ-^{32}P]ATP, only subunit VIa of COX from bovine heart and kidney was labelled [24].

In the present investigation the binding of TNP-ATP and [^{35}S]ATPαS to isolated COX from bovine heart and liver and from *Paracoccus denitrificans* was studied by measuring the fluorescence change of TNP-ATP or the bound radioactivity after equilibrium dialysis, respectively. In addition photoaffinity-labelling of isolated COX by 8-azido-[γ-^{32}P]-ATP is shown. The results indicate specific labelling of 6 or 7 subunits in the mammalian and only one subunit in the bacterial enzyme.

Materials and Methods

Materials. [^{35}S]ATPαS (400 Ci/mmol, 10 mCi/ml) was obtained from Amersham, Braunschweig, 8-azido-[γ-^{32}P]-ATP from ICN Biochemicals, (40 Ci/mmol). 2'(3')-O-(2,4,6,-Trinitrophenyl)-ATP (TNP-ATP) was synthesized as described before [25]. The reaction product was purified on a Sephadex LH-20 column (Pharmacia) and characterised by infrared and NMR spectra and by paper chromatography.

Isolation of COX. COX was prepared from isolated mitochondria of bovine liver and heart as described before [26]. The two-subunit COX of *Paracoccus denitrificans* was isolated with Triton X-100 [27]. The final pellet was stored frozen at -80° C.

Fluorescence measurements. The binding of TNP-ATP to COX was measured by its fluorescence change at 535 nm on a Perkin Elmer 650-40 fluorescence spectrophotometer. The samples were excited at 408 nm (slit width of 8 nm), and the emission spectra were recorded between 450 and 650 nm (slit width of 5 nm) [24]. The sample cuvette contained 0.5 μM COX dissolved in 10 mM K-Hepes, pH 7.4, 0.005 % dodecylmaltoside. The reference cuvette contained the same constitutents without COX. The titration was performed by addition of increasing amounts of TNP-ATP to both cuvettes, and the fluorescence difference at 535 nm was determined.

Equilibrium dialysis with [^{35}S]ATPαS. The equilibrium dialysis was performed in a plexiglas apparatus, produced in the workshop of the institute, containing two halfspheres of 100 μl volume, separated by a dialysis membrane. Each chamber contained in 90 μl 10 mM K-Hepes, pH 7.4, 100 mM KCl, 1 % Tween 20. One chamber contained 5 μM COX; into both was

added the indicated amount of [^{35}S]ATPαS (2-100 nCi). After dialysis for 72 hours at 4°C with gentle shaking, 50 μl were taken from each chamber and counted in a scintillation counter.

Photoaffinity-labelling by 8-azido-[γ-^{32}P]ATP. COX of bovine heart (6 μM), dissolved in 20 μl 100 mM K-Hepes, pH 7.2, 50 mM KCl, 0.7 % Na-cholate, was incubated in a small vessel of 10 mm diameter, covered with a glass plate, for 20 min at 0°C with 8-azido-[γ-^{32}P]ATP (2x10^6 dpm) and the indicated additions. Illumination was carried out at 0°C for 30 min at 350 nm wavelength with a Camag UV lamp, type TL 900, 8 W at 4 cm distance. SDS-PAGE was performed as described before [17].

Results and Discussion

In previous studies the binding of TNP-ATP to COX of bovine heart was followed by recording its visible absorbance change at 510 nm. Two binding sites with a K_d of 1.6 μM were obtained [25]. In the present investigation the fluorescence change of TNP-ATP was measured at 535 nm. As shown in Fig. 1, titration of COX from bovine heart and liver with increasing concentrations of TNP-ATP revealed almost identical binding curves. In contrast the two-subunit COX of *Paracoccus* showed saturation at a lower level. Digestion of COX from bovine heart with trypsin, which cleaves mainly subunits VIa and VIb, and to a lesser extent subunit IV, VIIa and VIIb [28], resulted in a binding curve similar to that of COX from *Paracoccus*. From the Scatchard plots of Fig. 2, three high-affinity binding sites are obtained for the enzymes of bovine heart and liver with dissociation constants K_d of 0.2 and 0.9 μM (heart) and 0.25 and 0.9 μM (liver), respectively. The number of binding sites (i.e. mole TNP-ATP per mole COX) were estimated by extrapolation of the initial part of the titration curves, where it is assumed that all added substrate is bound, and the saturation phase, as done in previous publications [25,29]. Our results contrast with those of previous studies, where one [14] or two binding sites were found [25]. In the former case no titration has been done, while in the latter case COX could have interfered with the visible spectrum of TNP-ATP. For the bacterial two-subunit enzyme one binding site with a K_d of

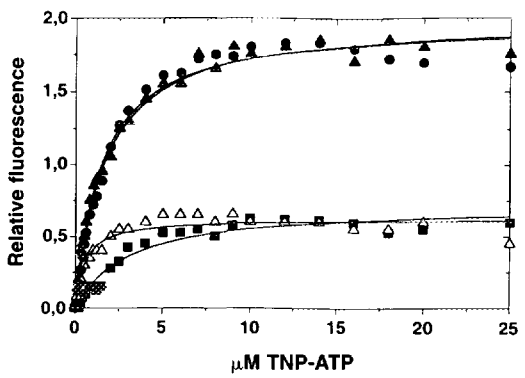

Fig. 1. Binding of TNP-ATP to COX of bovine heart, before and after treatment with trypsin, of bovine liver, and to the two-subunit COX of *Paracoccus denitrificans*. The binding was determined from the fluorescence change of TNP-ATP in the presence of COX. Closed triangles: bovine heart; closed circles: bovine liver; closed squares: *Paracoccus*; open triangles: trypsin-treated COX from bovine heart.

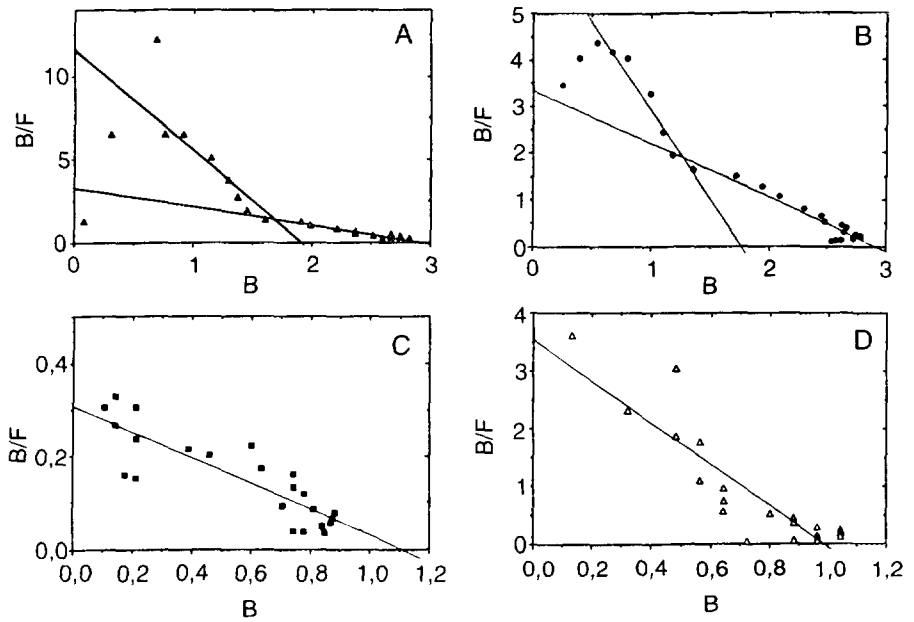

Fig. 2. Scatchard plots of the binding of TNP-ATP to COX of bovine heart, before and after trypsin treatment, of bovine liver, and to the two-subunit COX from *Paracoccus denitrificans*. The data were taken from Fig. 1. A: bovine heart; B: bovine liver; C: *Paracoccus*; D: trypsin-treated COX from bovine heart. Lettering of coordinates: B, bound TNP-ATP (mole per mole COX); F, free TNP-ATP (μM).

3.6 µM was found. Digestion of COX from bovine heart with trypsin resulted in one binding site with a K_d of 0.3 µM.

Derivatives of ATP may not bind to all nucleotide binding sites. Therefore we studied the binding of [^{35}S]ATPαS to the isolated enzymes by equilibrium dialysis, as shown in Fig. 3. In these studies cryptic nucleotide binding sites, which are only slowly exchanged, as shown e.g. for the uncoupling protein of brown adipose tissue mitochondria [30], were also determined, because dialysis was performed for 72 hours at 4° C. Again, for all three enzymes saturation curves were

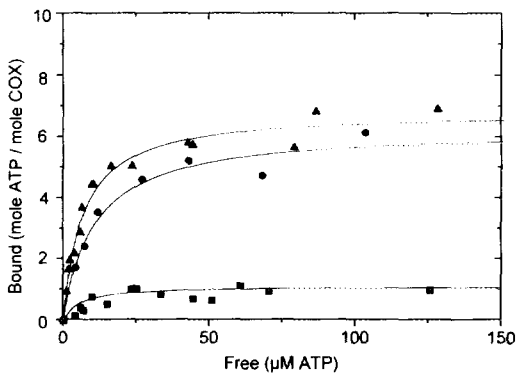

Fig. 3. Binding of [^{35}S]ATPαS to COX of bovine heart, bovine liver, and to the two-subunit COX of *Paracoccus denitrificans*. The binding was measured by equilibrium dialysis as described under Methods.

obtained at low ATP concentrations. But the curves clearly show differences between the heart and liver enzyme and in particular between the mammalian and bacterial enzyme. The Scatchard plot of Fig. 4 revealed almost straight lines with 7 and 6 binding sites and dissociation constants K_d of 7.5 and 12 μM for the bovine heart and liver enzyme, respectively. These dissociation constants apparently represent average values, because the Scatchard plot does not allow estimation of individual binding sites if there are more than two. For COX of *Paracoccus*. only one binding site with a K_d of 16 μM is calculated. These low K_d values indicate specific binding, and contrast with previously determined apparent K_d's for ATP of 2-5 mM [15] or 0.2 mM [16] at COX of bovine heart. The K_d values are summarized in table 1.

To verify the occurrence of multiple binding sites for ATP in mammalian COX, we labelled the enzyme of bovine heart with 8-azido-[γ-^{32}P]-ATP. In Fig. 5 is shown the Coomassie blue staining (A) and autoradiography (B) of COX, photoaffinity-labelled with 8-azido-[γ-^{32}P]-ATP at increasing concentrations of unlabelled 8-azido-ATP, after separation by SDS-PAGE. With increasing concentrations of unlabelled 8-azido-ATP the unspecific labelling of impurities above subunit II, including the position of subunit I, and between subunits IV and Va disappears and at 5-10 mM 8-azido-ATP only subunits II, IV, Va (and/or Vb), VIa, VIb or VIc, VIIa and VIIb or VIIc remain labelled (Fig. 5B, lanes 7 and 9). The labelling of these seven subunits corresponds with the number of seven bindings sites, as measured by equilibrium dialysis.

The function of the seventh ATP binding site in COX of bovine heart, not found in COX of bovine liver, has been suggested to be located at the N-terminal domain of subunit VIa-H (heart-type) and to influence tissue-specific energy transduction and rate of electron transport, depending on the matrix ATP/ADP ratio [20-22]. The remaining six high-affinity binding sites for

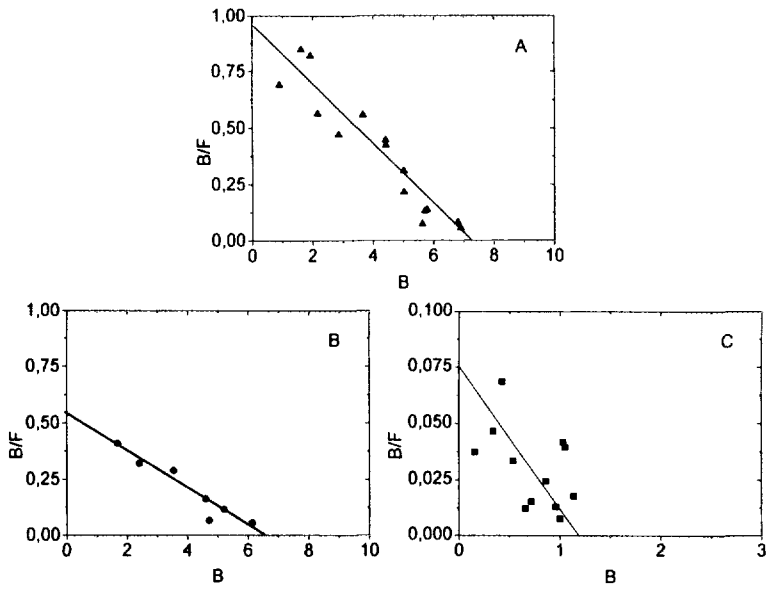

Fig. 4. Scatchard plots of the binding of [^{35}S]ATPαS to COX of bovine heart, bovine liver and to the two-subunit COX of *Paracoccus denitrificans*. The data were taken from Fig. 3. A: bovine heart; B: bovine liver; C: *Paracoccus*. Lettering of coordinates: B, bound ATP (mole per mole COX); F, free ATP (μM).

Table 1. Dissociation constants and number of binding sites for [^{35}S]ATPαS and TNP-ATP at COX from bovine heart, before and after trypsin treatment, from bovine liver and from the two-subunit COX of *Paracoccus denitrificans*. The data were calculated from the Scatchard plots of figures 2 and 4.

	K_d (μM)	number of binding sites
TNP-ATP		
Bovine heart	0.2 (2x), 0.9 (1x)	3
Bovine heart, trypsin-treated	0.3	1
Bovine liver	0.25 (2x), 0.9 (1x)	3
Paracoccus denitrificans	3.6	1
[^{35}S]ATPαS		
Bovine heart	7.5	7
Bovine liver	12	6
Paracoccus denitrificans	16	1

ATP in COX from heart and liver, and only one in bacterial COX (probably at subunit II) remain to be investigated. Preliminary data indicate the binding of both ATP and ADP with similar affinities at these binding sites [22]. The bound nucleotides could thus transmit to the enzyme the energy state (i.e. the ATP/ADP-ratio) within the cytoplasm and the mitochondrial matrix, since the nucleotide binding sites are located on both sides of the membrane. This follows from labelling of only subunits II, IV and VIIa (or VIIbc) of COX from bovine heart with 8-azido-[γ–^{32}P]-ATP after reconstitution in liposomes [17].

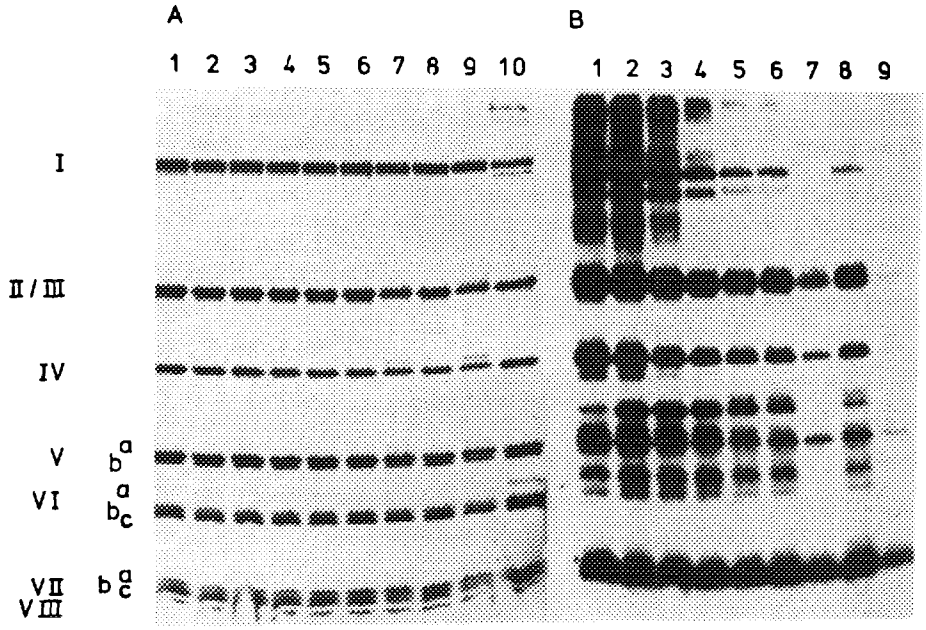

Fig. 5. Photoaffinity-labelling of COX from bovine heart by 8-azido-[γ–^{32}P]ATP at increasing concentrations of 8-azido-ATP. COX (6 μM) was illuminated as described under Methods with constant amounts of labelled but increasing concentrations of cold 8-azido-ATP. Lanes 1 and 2, 0.2 μM; lanes 3-9, 10 μM, 100 μM, 400 μM, 750 μM, 5 mM, 1 mM, 10 mM 8-azido-ATP, respectively, without (lane 1) or with the addition of 10 mM ATP (lanes 2-9).

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References

1. Iwato, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660-669.
2. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1995) Science 269, 1069-1074.
3. Haltia, T. (1990) Biochemistry 33, 9731-9740.
4. Kadenbach, B. and Merle, P. (1981) FEBS Lett. 135, 1-11.
5. Kadenbach, B., Kuhn-Nentwig, L. and Büge, U. (1987) Curr. Top. Bioenerg. 15, 113-161.
6. Capaldi, R.A. (1990) Ann. Rev. Biochem. 59, 569-596.
7. Kadenbach, B. (1986) J. Bioenerg. Biomembr. 18, 39-54.
8. Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104-1115.
9. Roberts, H. and Hess, B. (1977) Biochim. Biophys. Acta 462, 215-234.
10. Hüther, F.-J. and Kadenbach, B. (1986) FEBS Lett. 207, 89-94.
11. Malatesta, F., Antonini, G., Sarti, P., and Brunori, M. (1987) Biochem. J. 248, 161-165.
12. Hüther, F.-J., and Kadenbach, B. (1987) Biochem. Biophys. Res. Commun. 147, 1268-1275.
13. Hüther, F.-J., and Kadenbach, B. (1988) Biochem. Biophys. Res. Commun. 153, 525-534.
14. Lin, J., Wu, S. and Chan, S.I. (1995) Biochemistry 34, 6335-6343.
15. Bisson, R., Schiavo, G., and Montecucco, C. (1987) J. Biol. Chem. 262, 5992-5998.
16. Antonini, G., Malatesta, F., Sarti, P., Vallone, B., and Brunori, M. (1988) Biochem. J. 256, 835-840.
17. Reimann, A., Hüther, F.-J., Berden, J.A., and Kadenbach, B. (1988) Biochem. J. 254, 723-730.
18. Konstantinov, A., Vygodina, T., Popova, E., Berka, V., and Musatov, A. (1989) FEBS Lett. 245, 39-42.
19. Rigoulet, M., Guerin, B. and Denis, M. (1987) Eur. J. Biochem. 168, 275-279.
20. Anthony, G., Reimann, A., and Kadenbach, B. (1993) Proc. Natl. Acad. Sci. USA 90, 1652-1656.
21. Rohdich, F., and Kadenbach, B. (1993) Biochemistry 32, 8499-8503.
22. Kadenbach, B., Barth, J., Akgün, R., Freund, R., Linder, D. and Possekel, S. (1995) Biochim. Biophys. Acta 1271, 103-109.
23. Montecucco, C., Schiavo, G., and Bisson, R. (1986) Biochem. J. 234, 241-243.
24. Taanman, J-W., Turina, P. and Capaldi, R.A. (1994) Biochemistry 33, 11833-11841.
25. Reimann, A., and Kadenbach, B. (1992) FEBS Lett. 307, 294-296.
26. Kadenbach, B., Stroh, A., Ungibauer, M., Kuhn.Nentwig, L., Büge, U., and Jarausch, J. (1986) Methods Enzymol. 126, 32-45.
27. Ludwig, B. (1986) Methods Enzymol. 126, 153-159.
28. Capitanio, N., Peccarisi, R., Capitanio, G., Villani, G., De Nitto, E., Scacco, S. and Papa, S. (1994) Biochemistry 33, 12521-12526.
29. Garboczi, D.M., Hullihen, J.H., and Pedersen, P.L. (1988) J. Biol. Chem. 263, 15694-15698.
30. Huang, S.-G. and Klingenberg, M. (1995) Eur. J. Biochem. 229, 718-725.