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Stoichiometric binding of 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′- triphosphate to bovine heart cytochrome *c* oxidase

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The binding of 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) to isolated bovine heart cytochrome *c* oxidase (COX) was studied by following its specific spectral change at 510 nm. The quantitative titration revealed two binding sites for TNP-ATP per monomer COX with a K_d of 1.6 μM.

Cytochrome *c* oxidase; TNP-ATP; Nucleotide binding; Allosteric effector

1. INTRODUCTION

Mammalian cytochrome *c* oxidase (COX) contains 3 catalytic on mitochondrial DNA encoded subunits and 10 nuclear coded subunits which are absent in bacterial COX [1,2]. The nuclear coded subunits were suggested to bind allosteric effectors like nucleotides, hormones and second messengers and to modify the rate of electron transport and the efficiency of energy transduction [1,3]. The activity of membrane bound COX was shown to be influenced by ATP either acting from the cytosolic [4–11] or from the matrix side [12,13]. When acting from the cytosolic side it was not possible to distinguish between an interaction of ATP with the substrate or with the enzyme, because specific binding sites for ATP have been identified at cytochrome *c* [14,15].

The allosteric effect of nucleotides was verified, however, by the increased K_m for cytochrome *c* after photoaffinity labeling of bovine heart COX with 8-azido-ATP [6], and by the opposite effects of intraliposomal ATP and ADP on the K_m for cytochrome *c* of reconstituted COX from bovine heart [12], which were not obtained with COX from *Paracoccus denitrificans* [13], lacking the nuclear coded subunits.

A specific photoaffinity labeling of subunits IV and VIII of bovine heart COX by 8-azido-[γ-^{32}P]ATP [16] or [^{32}P]ATP [10] was described, but this could not be corroborated in a later study, where most subunits were shown to be labeled by 8-azido-[γ-^{32}P]ATP [7]. Thus at present neither the number of binding sites for ATP nor the involved subunits in the COX complex are known.

In the present study the binding of TNP-ATP to COX from bovine heart was investigated, and a stoichiometry of two TNP-ATP per monomeric COX was found. TNP-ATP has been previously applied as a coloured and fluorescent derivative of ATP to demonstrate its specific binding to heavy meromyosin ATPase [17], mitochondrial ATP-synthase [18–20] and H^+,K^+-ATPase [21] by its characteristic change of absorption or fluorescence.

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2. MATERIALS AND METHODS

Trinitrobenzene sulfonic acid (TNBS) was obtained from Serva and ATP from Boehringer. Sephadex LH-20 was purchased from Pharmacia. All other reagents were of the highest purity grade commercially available.

Bovine heart COX was prepared from isolated mitochondria as described before [22]. The synthesis of TNP-ATP was performed by pH-controlled trinitrophenylation of 2′- or 3′-OH of ATP by TNBS as described by Hiratsuka and Uchida [17]. The reaction product was purified on a Sephadex LH-20 column and characterized by infra-red and NMR spectra.

Absorption spectra of TNP-ATP were carried out in 10 mM K-HEPES, pH 7.4, and 0.05% dodecylmaltoside with an Uvikon 810 spectrophotometer (Kontron) as absolute spectra in the presence or absence of COX and as difference spectra by using tandem cuvettes to eliminate the absorption of the enzyme.

3. RESULTS AND DISCUSSION

Addition of stoichiometric amounts of isolated COX from bovine heart, dissolved in dodecylmaltoside, to TNP-ATP results in specific binding, as shown by the bathochromic change of the visible spectrum (Fig. 1). The maximum of the TNP-ATP spectrum at 470 nm is shifted to about 480 nm, after addition of COX. Similar spectral changes were obtained by binding of TNP-ATP to heavy meromyosin ATPase [17] and to soluble beef heart ATPase (F_1) [18].

The spectral change of TNP-ATP upon binding to

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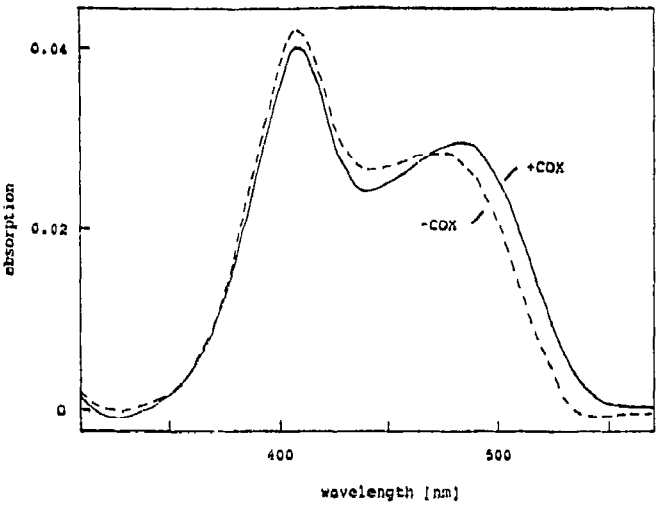

Fig. 1. Change of the visible spectrum of TNP-ATP upon binding to COX. The absolute spectrum of 2 μM TNP-ATP in 10 mM K-HEPES, pH 7.4, 0.05% dodecylmaltoside (---) and the spectrum after addition of 2 μM COX to the sample and reference cuvette (———) is presented.

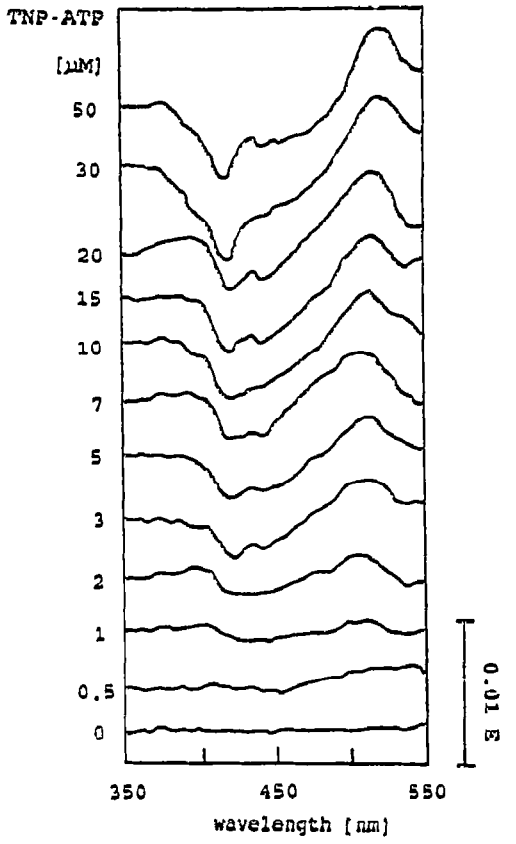

Fig. 2. Titration of COX with TNP-ATP. Difference spectra of TNP-ATP are shown. The measurements were performed with tandem cuvettes, containing TNP-ATP and COX either in the same (sample) or in separate compartments (reference). The two compartments of the cuvettes contained 10 mM K-HEPES, pH 7.4, 0.05% dodecylmaltoside, and one compartment of each cuvette contained 2 μM COX. The indicated amounts of TNP-ATP were added either to the COX containing compartment (sample) or to the COX-free compartment. Volume changes were corrected by buffer additions.

COX was analyzed quantitatively by titration of the enzyme with increasing concentrations of TNP-ATP. In order to exclude the absorption of COX in the same spectral region, we applied tandem cuvettes, as described in the legend to Fig. 2. The difference spectrum, obtained with increasing concentrations of TNP-ATP, as shown in Fig. 2, indicates a saturation of the spectral change. This saturation becomes more evident from Fig. 3, where the increase of the absorbance at 510 nm and at 510 nm minus 350 nm, taken from the difference spectra of Fig. 2, are plotted against the concentration of TNP-ATP. While at 510 nm a saturation of the spectral change is found, at 510 nm minus 350 nm two phases of absorbance increase are obtained. Stoichiometries (i.e. mol TNP-ATP per mol COX) were estimated by extrapolation from the initial part of the titration curves, where it is assumed that all added substrate is

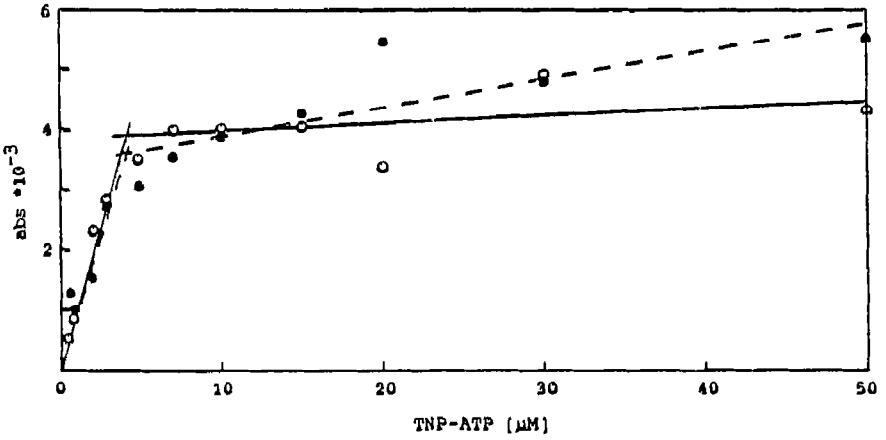

Fig. 3. Saturation of the spectral change of TNP-ATP upon binding to COX. The absolute absorbance difference at 510 nm (○——○) and the difference of the absorbance at 510 nm and 350 nm (●--●) are presented at increasing concentrations of TNP-ATP. The data were taken from Fig. 2. Stoichiometries (i.e. mol of TNP-ATP/mol of COX) were estimated by extrapolation from the initial part of the titration curve, where it is assumed that all substrate is bound, to the maximal absorbance change.

bound, and from the second (saturation) phase, as done in a previous publication [19]. The second phase of low affinity binding, obtained at 510 nm minus 350 nm, can be explained by the spectral change of COX at 350 nm, due to additional unspecific binding of TNP-ATP. In both cases a stoichiometry of 2 mol TNP-ATP per mol COX is obtained.

From Fig. 3, a K_d value of 1.6 μM is graphically determined for the binding of TNP-ATP to bovine heart COX. This K_d value for TNP-ATP, however, is probably lower than the value for non-derivatized ATP, which is expected to be in the millimolar range, i.e. the matrix nucleotide concentration range, because the trinitrophenyl-group will increase the affinity of ATP to the hydrophobic enzyme, as found previously for other enzymes [17,18,20].

In order to investigate how many binding sites for TNP-ATP exist at the outer cytosolic side of COX, we have titrated the enzyme with TNP-ATP after reconstitution in liposomes. Under these conditions no saturation of the spectral change at 510 nm was obtained (not shown), probably due to unspecific binding of TNP-ATP to liposomes.

The number of two TNP-ATP bound to solubilized COX from bovine heart, as found in this investigation, would be in accordance with the assumption of one binding site for ATP on the cytosolic and another one on the matrix side. The identity of the subunit(s) with which the nucleotide interacts, however, remains to be demonstrated.

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