INHIBITION OF MITOCHONDRIAL NUCLEOTIDE TRANSPORT AND PHOSPHORYLATION BY CIBACRON BLUE 3G-A

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1. Introduction

Inhibitors of mitochondrial reactions have become useful tools for investigating the mechanism of oxidative phosphorylation and the processes of active transport of metabolites, nucleotides or metal ions [1–3]. Cationic dyes of the cyanine type specifically inhibit NAD-linked respiration in both intact mitochondria and submitochondrial particles, with little or no effect on other mitochondrial functions [4–7]. In [8] a structure–inhibitor relationship of anthraquinone dyes was described with respect to the mitochondrial adenine nucleotide transport system.

Here, we confirm the specificity of interaction of Cibacron blue 3G-A with the ATP/ADP carrier system, and show the strong inhibition of mitochondrial NDP kinase, an enzyme located in the intermembrane space of liver organelles.

2. Materials and methods

2.1. *Chemicals*

All commercial nucleotides, substrates and coupling enzymes (a generous gift of Professor H. F. Schmidt) were products of Boehringer (Mannheim). Cibacron blue was from Ciba-Geigy (Basel). [^{14}C]ADP was a product of Radiochemical Center (Amersham). Nucleotide analogs ϵ-ADP and 8-BrIDP were synthesized as in [9,10].

Abbreviations: Cibacron blue, Cibacron blue 3G-A; 3G-A–Sepharose, Cibacron blue 3G-A–Sepharose; NDP kinase, nucleoside diphosphate kinase (EC 2.7.4.6); 8-BrIDP, 8-bromoinosine 5′-diphosphate; ϵ-ADP, 1,N^6 ethenoadenosine 5′-diphosphate

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2.2. *Biological preparations and analytical procedures*

The isolation of rat liver mitochondria, the measurement of mitochondrial respiration, the exchange of intramitochondrial ^{14}C-labeled adenine nucleotides with externally added ADP were done as in [11,12]. For the determination of dye binding to rat liver mitochondria, Cibacron blue (7–100 μM) was incubated with isolated mitochondria (1.2–1.5 mg protein/ml), in 3 ml media containing 0.15 M KCl, 25 mM Tris–HCl (pH 8.0) and 2 mM succinate. After 2 min incubation at room temperature, the mixtures were centrifuged at 20 000 × g for 5 min. The absorbance of the supernatants were measured at 610 nm, in 5 cm cuvettes. At >5 μM dye, appropriate dilutions were made. The dye concentrations were determined from an experimentally derived absorbance *vs* concentration curve, which was linear in the range used. A millimolar extinction coefficient of 13.6 at 610 nm was assumed for the monomeric dye [13]. Bound dye was calculated from the difference between free and total dye. Pig heart NDP kinase purified as in [14] was assayed colorimetrically as in [15]. The binding of Cibacron blue to purified NDP kinase was followed spectrophotometrically at 25°C. The difference spectra were recorded with a Specord UV–VIS instrument (K. Zeiss, Jena).

3. Results

3.1. *Effect of Cibacron blue on mitochondrial respiration*

As shown in [16,17] ϵ-ADP and AMP stimulate mitochondrial respiration only in the presence of externally added Mg^{2+}, and after a previous phosphorylation cycle of ADP to ATP. The stimulation of mitochondrial respiration through addition of ϵ-ADP

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or AMP after the state 3 to state 4 transition is due to the coupling of the respiratory chain-linked ADP phosphorylation to the reactions catalyzed by NDP kinase and adenylate kinase, both enzymes located on the outer side of the inner mitochondrial membrane. Cibacron blue over 5–10 nmol/mg protein inhibits significantly the stimulatory effect of ε-ADP on mitochondrial respiration, but is less effective on the stimulatory action of AMP or ADP. At higher dye/protein ratios (>20 nmol/mg protein) the effect of ε-ADP on mitochondrial respiration is almost abolished, whereas the stimulatory effect of AMP and ADP are seriously diminished (fig.1). ADP in excess as well as the uncouplers (FCCP at 1 μM) partly or completely restore the maximal rates of oxygen uptake of liver mitochondria. These results could be explained as follows:

(i) At low dye concentration, the activity of mitochondrial NDP kinase is selectively inhibited, whereas at higher concentrations both adenylate kinase and adenine nucleotide translocase are affected;

(ii) Cibacron blue has no direct access to the mitochondrial system of respiration and oxidative phosphorylation because of its negatively charged sulfonic groups.

The state 4 respiration was unaffected at all dye concentrations investigated, regardless of the nature of the substrate used.

The inhibitory effect of Cibacron blue upon mitochondrial respiration with different nucleotides shows a series of peculiarities:

(i) The drop in the respiratory control and adenylate kinase activity depends on the composition and the pH of the incubation medium, but is independent of the nature of the substrate. Thus, inhibition is stronger in sucrose than in KCl medium and at pH 7 rather than at pH 8. The inhibition of NDP kinase, as reflected by the reduced stimulation of respiration by ε-ADP, is less dependent on pH and composition of the incubation medium;

(ii) ATP at 1 mM in the respiration medium protects both the respiratory control and the activities of NDP kinase and adenylate kinase;

(iii) Bovine serum albumin (2 mg/ml) present in the respiration medium either before or after the addition of Cibacron blue prevents or completely reverses the inhibitory effects of the dye upon all the mitochondrial reactions investigated.

3.2. *Binding of Cibacron blue to rat liver mitochondria*

A Scatchard plot of bound and free Cibacron blue concentrations shows two distinct populations of binding sites (fig.2). The maximum binding capacity of rat liver organelles is equal to 113 nmol/mg protein. The 'high-affinity' binding sites have K_d 2 μM, where-

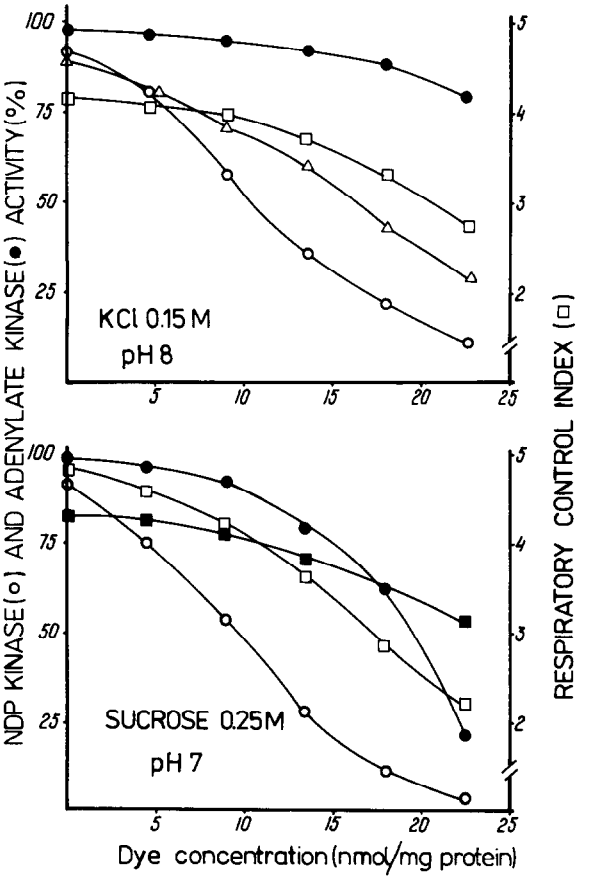

Fig.1. Effect of Cibacron blue on the respiratory control index (□), NDP kinase (○) and adenylate kinase (●) activity of intact rat liver mitochondria. The respiratory medium (0.5 ml final vol. 24°C) contained 250 mM sucrose, 25 mM Tris–HCl (pH 7), 10 mM potassium phosphate (pH 7), 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM glutamate, and 0–18 nmol Cibacron blue. When 0.15 M KCl replaced sucrose, the pH of the medium was adjusted to 8. The respiration was initiated with 0.8 mg mitochondrial protein; thereafter 120 nmol ADP, 50 nmol AMP or 110 nmol ε-ADP were injected. The activity of NDP kinase and adenylate kinase was calculated from the expression (ε-ADP or AMP rate – state 4 rate)/(ADP rate – state 4 rate) × 100, where the state 4 refers to the rate of respiration after the consumption of added ADP. (■) Respiratory control index determined in a medium supplemented with 1 mM ATP; (△) NDP kinase activity determined in a medium supplemented with 1 mM ATP.

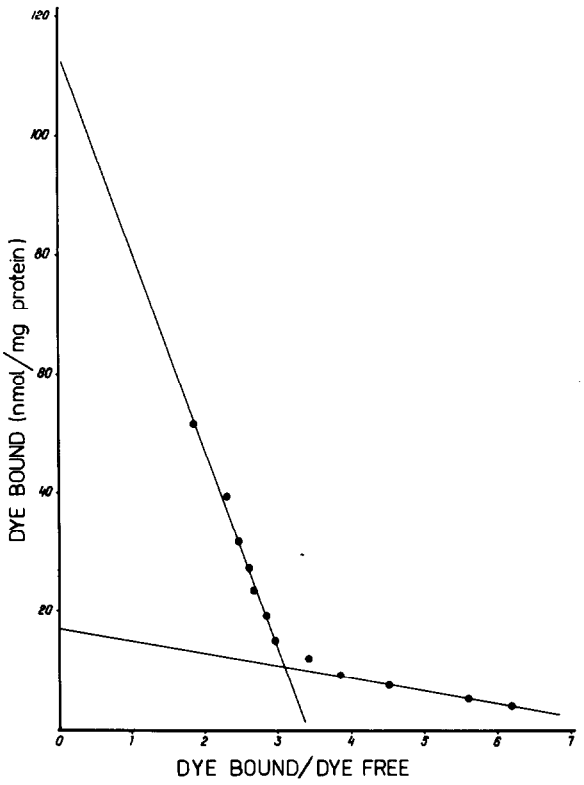

Fig.2. Binding of Cibacron blue to rat liver mitochondria. The experimental conditions are described in section 2.

as the 'low-affinity' binding sites have $K_d > 30$ μM. There are no significant differences among the affinities or the maximum binding capacities of rat liver mitochondria at pH 7 and 8, in KCl or sucrose medium. However, the incubation of mitochondria with nucleotides (2 mM ATP or ADP), inhibitors of transport or ADP phosphorylation (50 μM atractyloside, 2 μg oligomycin/mg protein), or uncouplers (1 μM FCCP), does not alter the binding of the dye. Only bovine serum albumin is capable of preventing the binding of the dye to mitochondria, in agreement with its protecting effect on mitochondrial respiration.

3.3. *Binding of Cibacron blue to purified NDP kinase*

Having indirectly demonstrated the inhibition of NDP kinase and adenylate kinase from mitochondrial respiration, we now turn our attention to the effect of dye upon the two partially purified enzymes [11]. Because lactate dehydrogenase is strongly inhibited by Cibacron blue [18], we had to determine adenylate kinase and NDP kinase activities colorimetrically, using pyruvate kinase as sole coupling enzyme (fig.3).

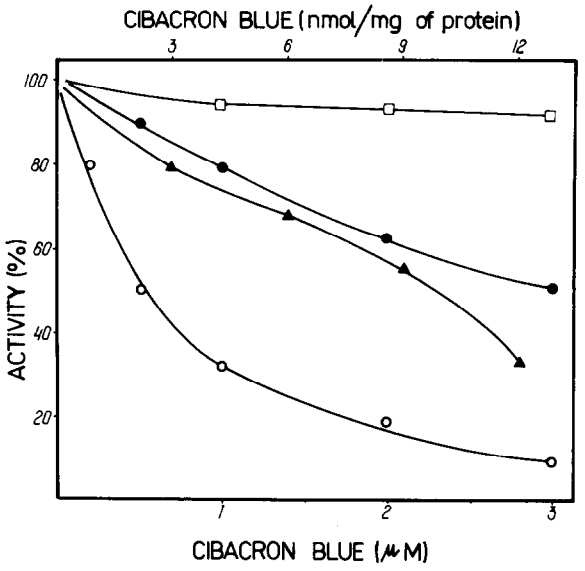

Fig.3. Inhibition of mitochondrial adenine nucleotide translocase (▲), NDP kinase (○), adenylate kinase (●) and coupling enzyme (rabbit muscle pyruvate kinase) (□) by Cibacron blue. Rat liver mitochondria (3 mg protein) pre-loaded with [^{14}C]-ADP were pre-incubated for 2 min at 2°C in 110 mM KCl, 20 mM Tris–HCl (pH 8), 1 mM EDTA, and 0–36 nmol Cibacron blue in 0.5 ml final vol. The reaction was triggered by addition of 10 nmol ADP. After 2 min incubation the reaction was stopped by addition of 50 nmol atractyloside; after centrifugation at 20 000 × g for 5 min, 0.2 ml aliquots of the supernatant were used for liquid scintillation counting. The activity of partially purified NDP kinase and adenylate kinase was measured colorimetrically exactly as in [15] using 0.5 mM ATP and 0.2 mM 8-BrIDP (or 0.2 mM AMP, respectively).

It is found that with 3 μM dye the activity of mitochondrial NDP kinase is inhibited by >90%. At the same dye concentration the activity of adenylate kinase is inhibited by 50%, while the coupling enzyme is inhibited by only 8% (this degree of inhibition does not affect the precision of the NDP kinase and adenylate kinase assay). The high affinity of the dye for NDP kinase is also demonstrated by the considerable alteration of its absorption spectrum upon binding as shown in fig.4. Practically all dye molecules are bound to the purified pig heart enzyme even at 1 μM Cibacron blue; an ATP concentration 300-times larger than this replaces a small fraction of Cibacron blue from the active site of NDP kinase. Thus, we could not determine the binding parameters of the dye to the purified NDP kinase, but we estimate K_d to be <0.1 μM.

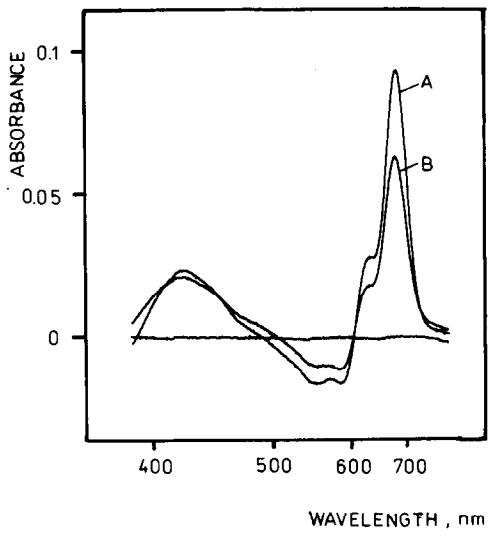

Fig.4. Difference absorption spectrum of Cibacron blue bound to purified pig heart NDP kinase. The sample cuvette (1 cm path-length) contained in 20 mM phosphate buffer (pH 7), 10 μM Cibacron blue and 28 μM NDP kinase (trace A) or 10 μM Cibacron blue, 28 μM NDP kinase and 3 mM ATP (trace B). The reference cuvette contained 10 μM Cibacron blue in the same buffer. NDP kinase concentration was expressed on the basis of the 17 500 M_r subunit.

4. Discussion

The interaction of Cibacron blue with the mitochondrial system of oxidative phosphorylation should be discussed in terms of 2 aspects:

(i) The translocation of the dye across the mitochondrial membranes;

(ii) The specificity of interaction with various enzymatic systems.

The inhibition of NDP kinase and adenylate kinase, as well as of the adenine nucleotide translocase (fig.3), seems to imply that the dye can easily cross the outer mitochondrial membrane but cannot reach the matrix space. The translocation of anions through the inner mitochondrial membrane is highly selective, therefore it would be important to know if minute amounts of Cibacron blue could penetrate the membrane by other means; under such circumstances it would no longer be possible to differentiate between the inhibition of ADP transport and ADP phosphorylation. However, the fast recovery of the respiratory control upon addition of bovine serum albumin (which binds 2 dyes/protein molecule with an intrinsic K_d of 7 μM) provides strong evidence against the penetration of Cibacron blue into the matrix space of intact mitochondria. The binding of Cibacron blue to mitochondria in a biphasic fashion is not unexpected since many proteins bind the dye in this manner. The 'low-affinity' binding of Cibacron blue to mitochondria could represent non-specific (presumably ionic) interactions of the dye with basic domains on proteins. Unlike the anthracycline drugs which possess a related chromophore [19,20], Cibacron blue cannot cross the lipid bilayer by passive diffusion because its pK_a is well below the physiological pH thanks to the sulfonic substituents.

Cibacron blue strongly inhibits NDP kinase, but not adenylate kinase or mitochondrial translocase, in a pH- and ionic strength-independent fashion. The characteristic difference spectrum resulting from this interaction is particularly obvious when using purified enzyme.

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