Interaction of Cibacron Dyes with Dehydrogenases and Kinases

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Cibacronblue 3 G-A, Inhibition, Interaction, Structural Requirement, Kinases and Dehydrogenases

The inhibition of the activity of various kinases and dehydrogenases by Cibacronblue 3 G-A and its structural analogues relies on the interaction of the anthrachinone moiety as well as of the neighbouring phenyl group of the dye with the proteins. Binding is intensified by hydrophobic substituents at the latter ring system.

Introduction

In 1969 it was demonstrated [1] that pyruvate kinase binds to Blue Dextran 2000. In a study of the mechanism of this absorption it could be shown that the binding of the enzyme is due to an interaction of the enzyme with the blue dye of Blue Dextran 2000 and not with the dextran itself. This property was used for the purification of pyruvate kinase of yeast [1] and later on also applied to the extraction of human erythrocyte pyruvate kinase [2], phosphofructokinase [3, 4] and various other enzymes [5]. In order to simplify the purification procedure the properties of the dye were examined [5]. It was found that the blue dye (Cibacronblue 3 G-A) could be covalently fixed to an insoluble carrier and used successfully for affinity chromatography. Later, on the basis of enzymatic and spectrophotometric analysis it was suggested that the blue chromophore specifically binds to protein ligand sites of NAD-domains [6, 7]. In order to define more specifically the structural requirements for the binding of the dye, we have analysed the binding properties of a number of dyes of the Cibacron-group to a variety of cellular enzymes, and here we report our findings.

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Abbreviations: PK, Pyruvate Kinase; PFK, Phosphofructokinase; HK, Hexokinase; GK, Glycerokinase; LDH, Lactate Dehydrogenase; SDH, Sorbitol Dehydrogenase; GR, Glutathione Reductase; GlDH, Glutamate Dehydrogenase; MDH, Malate Dehydrogenase.

Materials and Methods

Materials

All chemicals used were p.a. grade and purchased from Boehringer Mannheim GmbH if not otherwise stated. Glucose, glycerol and fructose were obtained from E. Merck AG, Darmstadt. ATPase was prepared according to [8].

Cibacronblue 3 G-A (**I**), Cibacronbrilliantblue BR-P (**III**), and Sulfoparablue (**II**) were from Ciba-Geigy AG, Basel. Anthralanblue G (**IV**), Anthralanblue B (**VI**), Remazolbrilliantblue R (**IX**), and Remazolbrilliantblue B (**X**) were obtained from Farbwerke Hoechst AG, Frankfurt. Compound **V**, **VII**, and **VIII** were supplied by Bayer AG, Leverkusen (for structures see Table I).

Methods

Time dependency of inactivation:

For time dependency measurements lactate dehydrogenase (0.2 μg/ml) was incubated with Cibacronblue 3 G-A (0.05 mg/ml) in 50 mM potassiumphosphate, pH 7.5. Samples were taken in time intervals of 1 minute. Assays were carried out at pH 7.5 in 2 ml of potassiumphosphate (50 mM) NADH (0.25 mM) and pyruvate (1 mM) in an Eppendorf Photometer.

Inhibition studies:

Enzyme activities were measured using pH-stat method in an PHM 62 pH-meter together with autoburette ABU 13 and TTT 60 titrator from Radiometer Copenhagen. Assays were carried out in a total volume of 2 ml with 0.125 mg dye. About 1 U of enzyme was preincubated with the various dyes for 5 minutes. Enzyme activity was measured and compared to the original activity without dye. The reaction was started with substrate. 0.02 N NaOH was used for titration of kinases, 0.02 N HCl for dehydrogenases.

Assay for pyruvate kinase (rabbit muscle):
10 mM ATP, 15 mM $MgSO_4$, 50 mM KCl, pH 7.5, start with 5 mM phosphoenolpyruvate.

Assay for hexokinase (yeast):
10 mM ATP, 15 mM $MgSO_4$, pH 7.5, start with 5 mM glucose.

Assay for glycerokinase (Candida mycoderma):
10 mM ATP, 15 mM $MgSO_4$, pH 7.5, start with 5 mM glycerol.

Assay for phosphofructokinase (rabbit muscle):
10 mM ATP, 15 mM $MgSO_4$, pH 7.5, start with 5 mM fructose-6-phosphate.

Assay for ATPase (yeast):
200 mM KCl, 50% glycerol, start with 10 mM ATP, 15 mM $MgSO_4$, pH 7.5.

Assay for lactate dehydrogenase (rabbit muscle):
10 mM NADH, pH 7.5, start with 10 mM pyruvate.

Assay for sorbitol dehydrogenase (sheep liver):
10 mM NADH, pH 7.5, start with 10 mM fructose.

Assay for glutathione reductase (yeast):
10 mM NADH, pH 7.5, start with 5 mM oxidized glutathione.

Assay for glutamate dehydrogenase (beef liver):
10 mM NADH, pH 7.5, start with 10 mM α-ketoglutarate, 20 mM ammoniumacetate.

Assay for malate dehydrogenase (pig heart, mitochondrial):
10 mM NADH, pH 7.5, start with 10 mM oxaloacetate.

Results and Discussion

On addition of Cibacronblue 3 G-A to lactate dehydrogenase a decrease of enzyme activity is observed. The time dependency of the binding of the dye to the enzyme and its inactivation as a function of a non saturating concentration of the dye is shown in Fig. 1. It can be seen that the enzyme is rapidly inactivated during the first minute. Afterwards smaller changes in the enzyme activity occur in a rate approximating a first order process. This behaviour can be explained by a fast binding process leading to loss of activity and a following slow conformational change resulting in an additional slow inactivation.

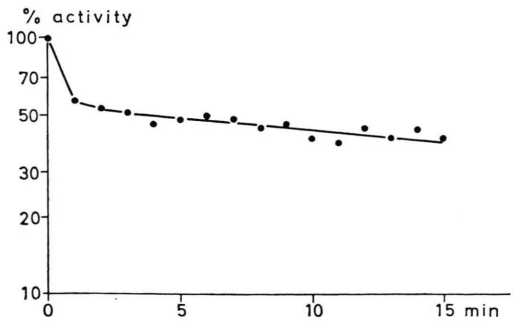


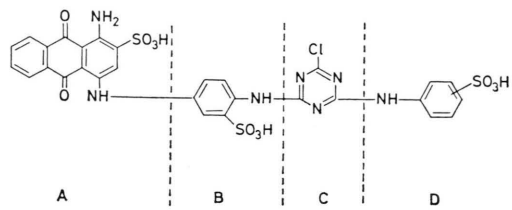
Fig. 1. Time dependency of inactivation of lactate dehydrogenase by Cibacronblue 3 G-A.

In order to define comparable conditions for the analysis of the structural requirement of the dyes, studies of the change of activity were carried out after 5 minutes incubation within the second phase of the inactivation process. This simplified procedure was furthermore justified by the unusual properties of the dye. Indeed it was found that the spectral behaviour of the dyes does not obey Lambert-Beer's law indicating association-dissociation equilibria, a property which could be confirmed by centrifugation studies demonstrating dye aggregation even at low concentrations. This phenomena was found to depend on the ionic strength as well as solvent composition of the system [9].

Table I summarizes the percental inhibition of various dehydrogenases as well as kinases after 5 minutes of incubation with Cibacronblue 3 G-A (**I**) and various analogues. The distribution clearly demonstrates the variety of binding properties of the enzymes with respect to the configuration of the dyes.

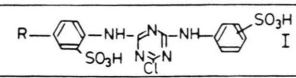
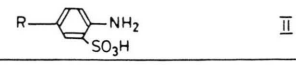
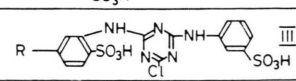
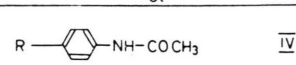
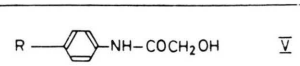
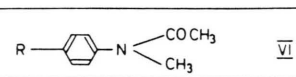
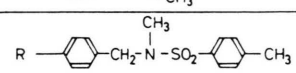
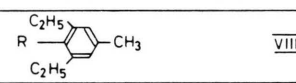
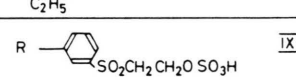
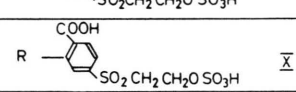
Cibacronblue 3 G-A (**I**) consists of four different ring systems (formula 1). A comparison of the configuration of the dyes with respect to inhibition properties demonstrates that parts C and D seem to be of minor importance for the interaction with the enzymes, and that the major function is located in the ring systems A and B.

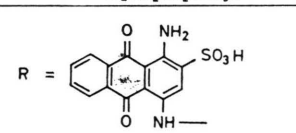
Of special interest is the dependency of the binding from the hydrophobicity of part B as illustrated



Formula 1. Cibacronblue 3 G-A.

Table I. Inhibition of enzymes by Cibacronblue 3 G-A and related dyes. 90–100% of original activity: ○; 10–90% of original activity: ×; less than 10% of original activity: ××.

PK	PFK	HK	GK	ATPase	LDH	SDH	GR	GlDH	MDH	
XX	XX	X	○	○	XX	○	X	X	○	 I
XX	XX	X	X	X	XX	○	X	X	X	 II
X	○	○	○	X	X	○	X	X	○	 III
X	○	X	○	X	○	○	X	X	○	 IV
XX	○	X	X	X	X	○	XX	X	○	 V
X	○	○	○	○	○	○	X	○	○	 VI
XX	XX	X	○	X	XX	○	X	X	XX	 VII
XX	XX	XX	XX	X	X	X	X	XX	X	 VIII
XX	X	X	○	X	X	○	XX	X	X	 IX
X	○	○	○	○	X	○	X	○	○	 X



by a comparison of the action of the dyes with an amino group in ring B (f. i. Sulfoparablue **II**, compound **III** – **VI**), similar to Cibacronblue 3 G-A, with more hydrophobic dyes (compound **VII** and **VIII**) indicating that the latter function increases the degree of binding to the enzymes, and consequently the inhibition. An optimized structure such as compound **VIII** strongly inhibits nearly all the enzymes tested. A detailed mechanistic analysis of this interaction is under current investigation.

Recently it has been suggested that the structural requiremens for the binding of Cibacronblue to enzymes rely on its specific configuration analogous to the structure of nicotinamide-adenine dinucleotide with ring C and D of the dye being analogous to the nicotinamide moiety, and the anthrachinone portion being analogous to the adenine system of NAD respectively NADH. Furthermore it was proposed that Cibacronblue 3 G-A specifically marks the NAD-domain of various enzymes [6, 7]. On the basis of the comparison of various structures reported here this suggestion might need reconsideration. Because of the strong binding activity of compound **VIII** we would rather prefer to see the analogy only with respect to the adenosine moiety of ATP, ADP, NAD and not of the dinucleotide as a whole, although further experimentation should support this view. In addition it is interesting to remember that a number of enzymes tested so far do not function enzymatically with NAD/NADH. Summarizing it can be stated that the compounds might well be suitable in a test for adenine nucleotide- as well as NAD-binding sites in a large variety of enzymes.

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