Gen. Physiol. Biophys. (1990), 9, 519—528

Interaction of Cibacron Blue 3GA and Remazol Brillant Blue R with the Nucleotide Binding Site of Lactate Dehydrogenase and $(Na^+ + K^+)$-ATPase

V. Ďurišová[1], A. Vrbanová[1], A. Ziegelhöffer[2] and A. Breier[1]

1 *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska* 5, 833 34 *Bratislava, Czechoslovakia*
2 *Institute of Heart Research, Slovak Academy of Sciences, Dúbravská cesta* 9, 842 33 *Bratislava, Czechoslovakia*

Abstract. The interaction of Cibacron Blue 3GA (C.I.2) and Remazol Brillant Blue R (C.I.19) with purified preparations of Lactate dehydrogenase (LDH) and $(Na^+ + K^+)$-ATPase was studied by means of the enzyme kinetics method. LDH was found to be inhibited by both C.I.2 and C.I.19, with the former being a stronger inhibitor. This may be explained by the fact that in contrast to C.I.19, C.I.2 resembles the whole molecule of the specific cofactor. $(Na^+ + K^+)$-ATPase activity was inhibited by both dyes to approximately similar degree. C.I.2 and C.I.19 resemble the ATP molecule to approximately similar extent, particularly as concerns the molecule shape and size. The results obtained confirmed the applicability of C.I.2 and C.I.19 as nucleotide-specific ligands.

Key words: $(Na^+ + K^+)$-ATPase — Lactate dehydrogenase — K^+-pNPPase — Cibacron Blue 3GA — Remazol Brillant Blue R

Introduction

Antraquinone dyes (Cibacron Blue 3GA — C.I.2 and Remazol Brillant Blue R — C.I.19) represent group-specific ligands with high affinities to nucleotide-binding sites of purine nucleotides. This specific property of C.I.2 and C.I.19 has been utilized in their applications as specific ligands in affinity chromatography of dehydrogenases such as lactate dehydrogenase (Gemeiner et. al. 1981), malate dehydrogenase (Scawen et al. 1982) or 6-phosphogluconate dehydrogenase (Kroviarski et al. 1988), and kinases like phosphofructo kinase (Kopperschläger et al. 1982), or phosphoglycerate kinase (Kulbe and Schuer 1979).

Besides the latter mentioned nucleotide-specific interactions antraquinone dyes are capable to provide also ionic- (Subramanian 1984) and hydrophobic

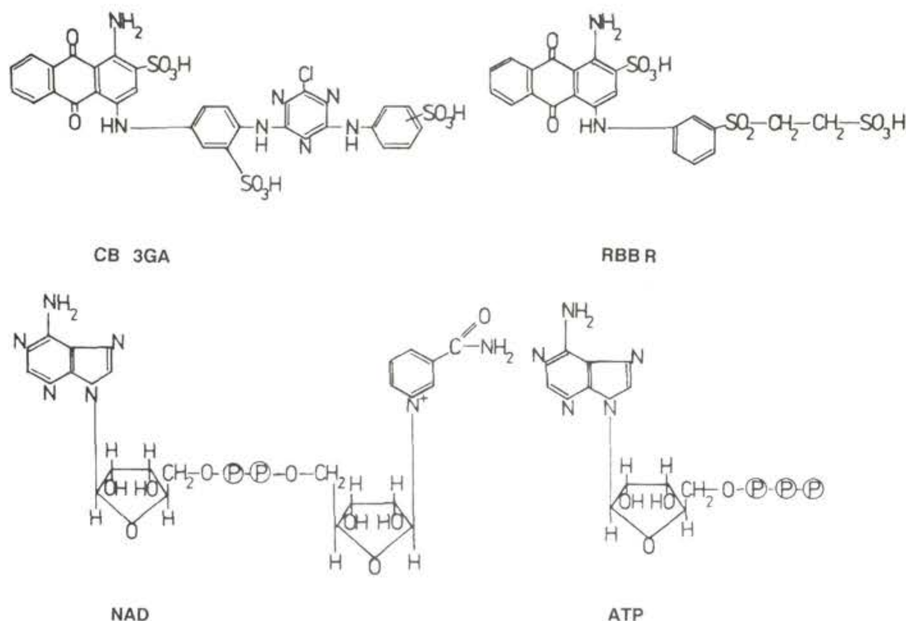

Fig. 1. Structures of reactive dyes C.I.2 and C.I.19 in comparison to ATP and NAD^+.

interactions (Breier et al. 1987a) with proteins. The diversity of the possible interactions of C.I.2 and C.I.19 offers them almost general reactivity which may manifest itself in binding of these dyes also to different proteins with not exclusively enzymatic nature (Miribel et al. 1988). Similarities in charge displacement as well as the aromatic nature are believed to be responsible for the nucleotide-specific properties of antraquinone dyes; indeed, due to their molecule size and shape as well as to the topology of π-orbitals C.I.2 and C.I.19 are structurally related to purine nucleotides (Thompson et al. 1975). The high flexibility of molecules of C.I.2 and C.I.19 makes these substances suitable to mimic the shape of other molecules (Fig. 1).

Experiments with kinases have revealed that C.I.2 and C.I.19 are interacting specifically with the latter enzymes in their ATP-binding sites (Kopperschläger and Johansson 1985). Neverthelles similar data concerning the ATPases have been not available as yet. In preliminary experiments (Ďurišová et. al. 1989) both dyes were found to effectively inhibit the activity of lactate dehydrogenase (LDH) from skeletal muscle as well as that of heart sarcolemmal $(Na^+ + K^+)$-ATPase. The aim of the present paper is quantification of the latter effect.

Materials and Methods

LDH from bovine skeletal muscle with a specific activity of 2.5 μmol $NAD^+ . mg^{-1} . min^{-1}$ was prepared by affinity chromatography according to the method of Gemeiner et al. (1987). LDH activity was estimated based on the rate of $NADH_2$ formation during conversion of lactate to pyruvate. Enzyme reaction was performed in 3 ml of reaction medium containing phosphate buffer (20 mmol . l^{-1}, pH = 7.5), L-lactate sodium salt, (1 mmol . $^{-1}$), NAD^+ (0.125—4.000 mmol . l^{-1}), hydrazine (1 mmol . l^{-1}) and 100 μg enzyme protein per ml. Reaction was started with addition of enzyme, and the rate of $NADH_2$ formation was measured spectrophotometrically at 340 nm utilizing the principle of Wartburg's optical test (Bergmeyer 1970).

Partially purified heart membrane fraction enriched on sarcolemma was isolated using the method of hypotonic shock combined with NaI treatment (Kostka et al. 1981). $(Na^+ + K^+)$-ATPase activity was established as the difference in amounts of inorganic phosphate liberated during ATP-hydrolysis in the presence of NaCl, KCl, and $MgCl_2$ (100, 10 and 2 mmol . l^{-1}) and in the presence of $MgCl_2$ only. Reaction was performed in a final volume of 0.5 ml containing imidazole-HCl buffer (25 mmol . l^{-1}, pH 7.0), ATP (0.125—2.000 mmol . l^{-1}), as well as the respective cations and it was started by addition of membrane fraction (final concentration 50 μg membrane protein per ml). After 10 minutes incubation at 37 °C the reaction was terminated by addition of 1 ml of trichloroacetic acid (0.73 mol . l^{-1}). Further details concerning the estimation of the $(Na^+ + K^+)$-ATPase activity as well as those concerning the characteristics of the membrane fraction applied were published elsewhere (Ziegelhöffer et al. 1983; Breier et al. 1988). Protein concentration was determined according to Lowry et al. (1953).

K^+-pNNPase activity was established as the difference in *p*-nitrophenyl (pNP) formed from *p*-nitrophenylphosphate (pNPP) in the presence and absence of KCl (5 mmol . l^{-1}) during 10 minutes reaction at 37 °C in a medium containing $MgCl_2$ (2 mmol . l^{-1}). Enzyme reaction was performed in a final volume of 0.5 ml imidazole-HCl buffer (25 mmol . l^{-1}, pH 7.0) with 50 μg of membrane protein per ml. Reaction was started with addition of pNPP (0.125—5.000 mmol . l^{-1}) and stopped by addition of 0.5 ml NaOH (0.1 mol . l^{-1}). Amounts of pNP formed were established spectrophotometrically at 410 nm. For more details see Breier et al. (1987b).

The activity of trypsin was established from the velocity of *p*-nitroaniline (pNA) formation from N^{α}-benzoyl-DL-arginine-*p*-nitroaniline (BAPA) within 10 minutes. Reaction conditions: 2.5 ml imidazole-HCl buffer (25 mmol . l^{-1}, pH 7.0), 25 °C, and BAPA 0.025—0.25 mmol . l^{-1}. Reaction was started by adding 50 mg per ml of membrane protein and it was terminated by addition of 0.5ml acetic acid (30 per cent). The amounts of pNA liberated were determined spectrophotometrically at 410 nm. Further details have been described in a previous paper (Gemeiner and Breier 1982).

Estimation of C.I.2 binding to isolated cardiac sarcolemma: Isolated membrane fraction (100 μg per ml) was incubated together with C.I.2 (1.25—20.00 mmol . l^{-1}) for one hour at 37 °C in the presence and absence of ATP (2 mmol . l^{-1}). The reaction was stopped by spunning down at 3000 × *g*, with subsequent resuspension in imidazole-HCl buffer (25 mmol . l^{-1}, pH 7.0) and repeated centrifugation at same *g*. The final pellet was dissolved in the same imidazole buffer as previously, supplemented with 5 per cent SDS. The amount of bound C.I.2 was monitored spectrophotometrically at 620 nm.

Enzyme kinetics and binding studies were evaluated using non-linear regression. The kinetic parameters obtained were characterized by 95 % confidence intervals of according to Rektorys (1968).

All chemicals were of analytical grade and they were purchased from Sigma (St. Louis) and Lachema (Brno).

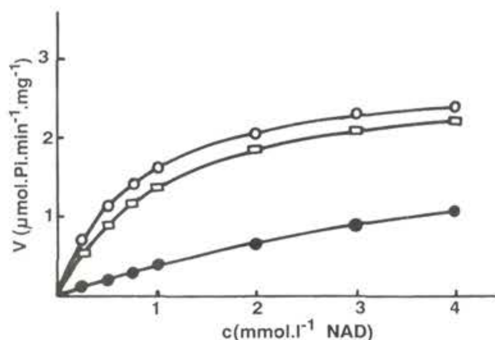

Fig. 2. Effect of NAD$^+$ on lactate dehydrogenase activity in the absence and presence of antraquinone dyes C.I.2 and C.I.19 ○ — control values; ● — C.I.2 ($5\,\mu$mol.l^{-1}); □ — C.I.19 ($5\,\mu$mol.l^{-1}). The dyes were left to interact with the enzyme directly in a medium for estimation of enzyme activity. Results represent mean values of 6 experiments.

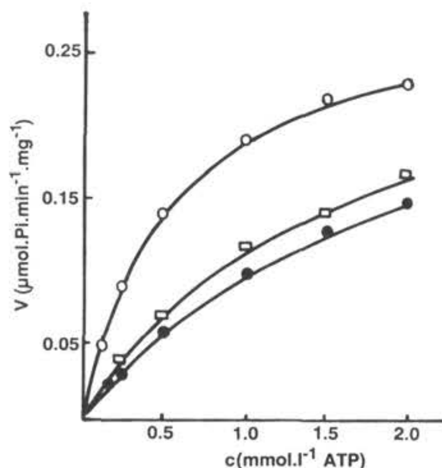

Fig. 3. Effect of ATP on ($Na^+ + K^+$)-ATPase activity in the absence and presence of antraquinone dyes C.I.2 and C.I.19. ○ — control values; ● — C.I.19 ($5\,\mu$mol.l^{-1}); □ — C.I.2 ($5\,\mu$mol.l^{-1}). The dyes were left to interact with the enzyme directly in a medium for the estimation of enzyme activity. Results represent mean values of 6 experiments.

Results and Discusion

C.I.2 and C.I.19, $5\,\mu$mol.l^{-1}, had inhibitory effects on the specific activity of LDH. The kinetics of this inhibition is illustrated in Fig 2. The curves in Fig. 2. were obtained by non-linear regression and they indicated that C.I.2 had a more

Table 1. Kinetic parameters of stimulation of LDH and $(Na^+ + K^+)$-ATPase by their specific nucleotide substrates in the absence and presence of antraquinone dyes C.I.2 and C.I.19.

Dye*	LDH		$(Na^+ + K^+)$-ATPase	
	K_M [μm . l^{-1}]	V_{max} [μmol . NAD^+ . min^{-1} . . mg^{-1}]	K_M [μmol . l^{-1}]	V_{max} [μmol . ATP . min^{-1} . . mg^{-1}]
none	0.55 ± 0.03	2.69 ± 0.12	0.33 ± 0.02	0.27 ± 0.01
C.I.2	6.24 ± 0.08	2.69 ± 0.09	1.45 ± 0.10	0.27 ± 0.01
C.I.19	0.82 ± 0.02	2.69 ± 0.14	1.72 ± 0.12	0.27 ± 0.01

* The dyes (5 μmol . l^{-1}) were applied directly into the medium for the estimation of enzyme activity.

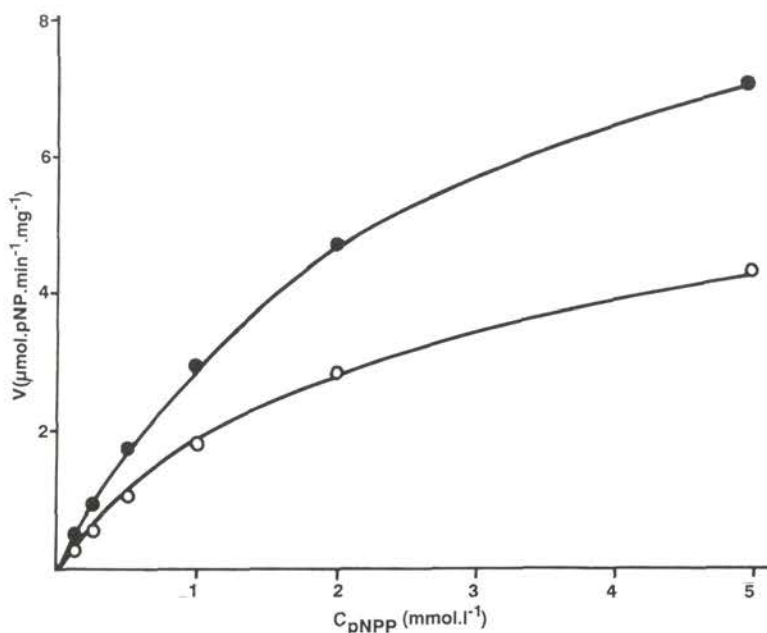

Fig. 4. Effect of pNPP on K^+-pNPPase activity in the absence and presence of antraquinone dye C.I.2. ● — control values; ○ — C.I.2 (5 μmol . l^{-1}). The dye was left to interact with the enzyme directly in a medium for the estimation of enzyme activity. Results represent mean values of 6 experiments.

pronounced inhibitory effect on LDH activity than did C.I.19. The respective kinetic parameters for both substances investigated are given in Table 1. and they suggest that the inhibition is of pure competitive nature. The above results are in good agreement with those reported by Mislovičová et al. (1988), with the LDH reaction kept running in direction of conversion of pyruvate to lactate.

Table 2. Kinetic parameters of stimulation of K^+-pNPPase and trypsin by *p*-nitrophenylphosphate and Na^{α}-benzoyl-DL-arginine-p-nitroanilide in the absence and presence of C.I.2.

	K^+-pNPPase		Trypsin	
	K_M [mmol . l^{-1}]	V_{max} [μmolpNP . h^{-1} . mg^{-1}]	K_M [mmol . l^{-1}]	V_{max} [μmolpNA . h^{-1} . mg^{-1}]
none	2.58 ± 0.12	10.79 ± 0.48	0.19 ± 0.01	11.22 ± 0.41
C.I.2	2.58 ± 0.17	6.54 ± 0.32	0.19 ± 0.01	11.22 ± 0.37

The dye (5 μmol . l^{-1}) was applied directly into the medium for the estimation of enzyme activity. pNP = *p*-nitrophenylphosphate; pNA – *p*-nitroaniline.

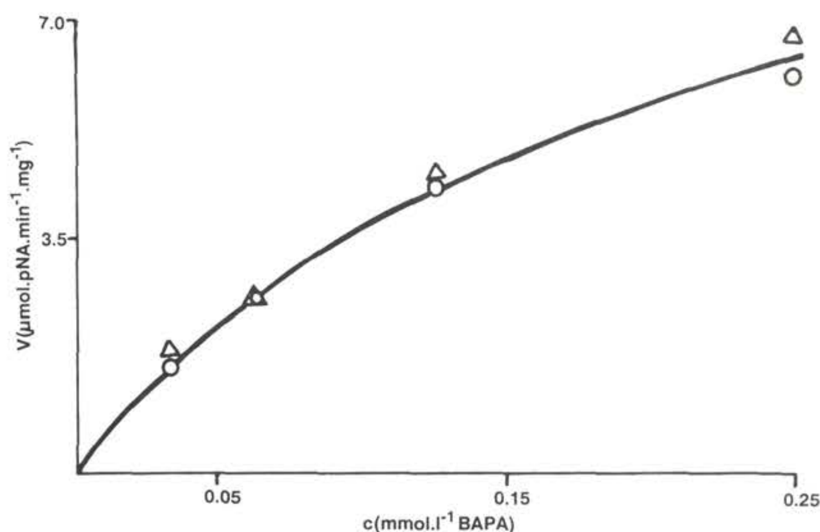

Fig. 5. Effect of BAPA on trypsin activity in the absence and presence of antraquinone dye C.I.2. ○ — control values; △ — C.I.2 (5 μmol . l^{-}). The dye was left to interact with the enzyme directly in a medium for the estimation of enzyme activity. Results represent mean values of 6 experiments.

The observation that C.I.2 is a more potent inhibitor of LDH reaction than is C.I.19 may be explained by the difference in their structure. From the latter aspect C.I.19 mimic the adenine moiety of $NADH_2$ molecule only, whereas C.I.2 may probably mimic the whole molecule of the dinucleotide.

In the presence of C.I.2 and C.I.19 the reaction of $(Na^+ + K^+)$-ATPase was also inhibited. The kinetics of this inhibition is shown in Fig. 3. Both substances investigated seem approximately similar potent inhibitors of the enzyme. The inhibition with both C.I.2 and C.I.19 was also found to be of nature competitive (Table 1). The minor difference observed in the K_m values of

Table 3. Adsorption isotherm parameters of C.I.2 binding to sarcolemmal membrane.

Binding	B_{max} [nmol . mg^{-1}]*	$C_{0.5}$ [μmol . l^{-1}]	Determination coefficient
Total	47.4 ± 1.6	11.57 ± 1.09	0.988
In the presence of ATP	37.9 ± 1.6	11.57 ± 0.99	0.992
Specific$^+$	9.5 ± 0.4	11.66 ± 0.77	0.998

* expressed in nmol of C.I.2. per mg membrane protein. The parameters were counted by nonlinear regression according to: $B = B_{max} . c . (c + C_{0.5})^{-1}$ (Breier et al. 1987a). Experimental data from Fig. 6 were used for the computations.

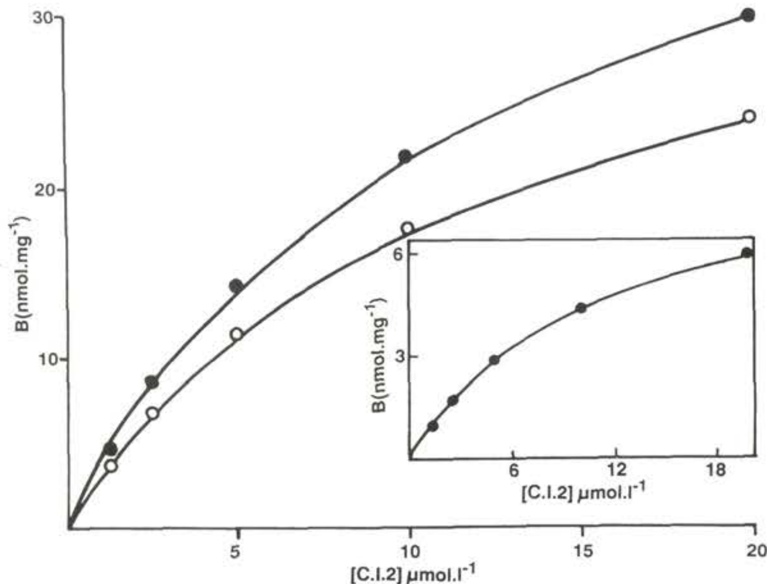

Fig. 6. Adsorption isotherms of C.I.2 binding to sarcolemmal membrane. Binding of C.I.2 in the absence (●) or presence (○) of ATP (2 mmol . l^{-1}). Insert: Specific binding of C.I.2 expressed as the difference between the amounts of the dye bound to membrane in the absence and presence of ATP.

inhibition of $(Na^+ + K^+)$-ATPase between C.I.2 and C.I.19 also indicates that in this case the diversity in shape and size of the inhibitor molecules do not play a dominant role.

The inhibition of K^+-pNNPase by C.I.2 (Fig. 4, Table 2) was non-competitive indicating that the active site of K^+-pNNPase is different from that of the $(Na^+ + K^+)$-ATPase, although both enzymes are associated with a

Na-pump (Vyskočil et al. 1981; Breier et al. 1987b). Ionic bounds as well as hydrophobic bounds may both participate on the interactions of C.I.2 with the K^+-pNNPase. Another alternative is also plausible: C.I.2 may inhibit of K^+-pNNPase activity via interaction and chemical modification of the part of the enzyme molecule responsible for $(Na^+ + K^+)$-ATPase activity.

The same concentrations of C.I.2 added to K^+-pNNPase, had no effect on the activity of trypsin (Fig. 5, Table 2).

C.I.2 may bind to sarcolemma membranes both specifically and non-specifically. Specific may be considered the binding which occurs in the nucleotide-binding sites, i.e. that in competitive relationship to nucleotide binding and related to inhibition of $(Na^+ + K^+)$-ATPase activity. An analysis of concentration-dependent binding of C.I.2 to isolated sarcolemmal membranes revealed that ATP counteracts significantly the specific interaction of C.I.2 with the membranes (Fig. 6, Table 3). However, a relatively significant amount of C.I.2 is bound to the sarcolemmal membranes in loci other than the nucleotide-binding sites. This binding considered as non-specific probably involves hydrophobic or ionic interactions and is related to high partition coefficient of C.I.2 in the membrane-water system.

From the results obtained the following conclusions may be drawn: a) the antraquinone dyes C.I.2 and C.I.19 inhibit those enzymes only which contain some nucleotide-binding sites;

b) if the nucleotide-binding site on the respective enzyme is identical with its active site, similarly as in the case of LDH or $(Na^+ + K^+)$-ATPase, a competitive inhibition occurs;

c) if antraquinone dyes interact with a nucleotide-binding site other than the substrate-binding site of the respective enzyme non-competitive inhibition may be observed;

d) comparing the results obtained with LDH and $(Na^+ + K^+)$-ATPase inhibition by C.I.2 and C.I.19 it may be concluded that both inhibitors have really a nucleotide specific character and they will act anywhere, where they can replace an adenine nucleotide and/or a nicotineamide-adenine dinucleotide;

e) a further conclusion to be drawn is that the inhibitory effect of the dyes investigated is the more expressed the more their molecule resemble in shape and size the substrate to be replaced;

f) the results obtained confirmed the suitability of the antraquinone dyes investigated for use as nucleotide-specific ligands in enzyme reactions as well as in some special cases of affinity chromatography.

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Final version accepted May 15, 1990