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Interaction of lactate dehydrogenase with anthraquinone dyes: characterization of ligands for dye–ligand chromatography

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Abstract

Anthraquinone dyes (ADs), originally developed for the textile industry, are useful nucleotide-specific ligands for the purification of proteins by affinity techniques. Their specific feature is to mimic the adenine nucleotides ATP, ADP, NAD, NADH, which enables them to interact with the nucleotide-binding sites of enzymes such as dehydrogenases, kinases and ATPases. In the present study, the interactions and/or inhibitory effects of seven ADs, including Cibacron Blue F3G-A, Remazol Brilliant Blue R, on the activity of lactate dehydrogenase (LDH) were investigated. The ADs used in this paper could be divided into two groups: (i) AD1–AD3 which do not contain a triazine moiety; (ii) AD4–AD7 which contain the triazine moiety. Enzyme kinetics and zonal affinity chromatography were used for the characterization of the interaction affinity between the dye and LDH. Enzyme kinetic measurements were carried out at three different pH values: 6.5, 7.5 and 8.5. The relationship between physical and chemical properties of ADs (e.g., acid–basic properties, three dimensional structure of the respective dyes) and their interaction efficiency with LDH was studied. LDH activity was inhibited by all ADs, excluding AD1 (precursor of the blue dyes) and inhibition was always competitive. Similarity in the mutual position of the acidic and basic groups in NADH and the respective AD molecule was found to be a crucial factor for influencing the inhibitory action of the substance. The existence of ADs in the protonated form should be considered as another factor, important for the ADs inhibitory action on this enzyme.

Keywords: NADH-binding-site; Dye-ligand chromatography; Anthraquinone dyes; Lactate dehydrogenase; Enzymes

1. Introduction

Anthraquinone dyes (ADs), including the Reactive Blue 2 (Cibacron Blue F3G-A, AD7; see Fig. 1) are generally recognized as ligands with highly flexible molecules and expressing nucleotide-mimetic properties [1,2]. The latter properties of ADs have been utilized in the purification of several dehydrogenases and kinases by bioaffinity separation techniques [3–8]. ADs were also found to inhibit numerous nucleotide-dependent and nucleotide-utilizing enzymes such as the lactate dehydrogenase (LDH, [9]), NAD-P-dependent carbonyl reductase [10], hexokinase [11] and the Na/K-ATPase [9,12]. In the case of formate dehydrogenase, it is precisely the anthraquinone moiety of the dyes, which, by means of electrostatic interactions, is capable of recognizing the NADH-binding site of the enzyme [13].

The use of the nucleotide-mimetic and biomimetic properties of ADs and particularly that of AD7 may be manyfold: (i) via the blockade of adenosine

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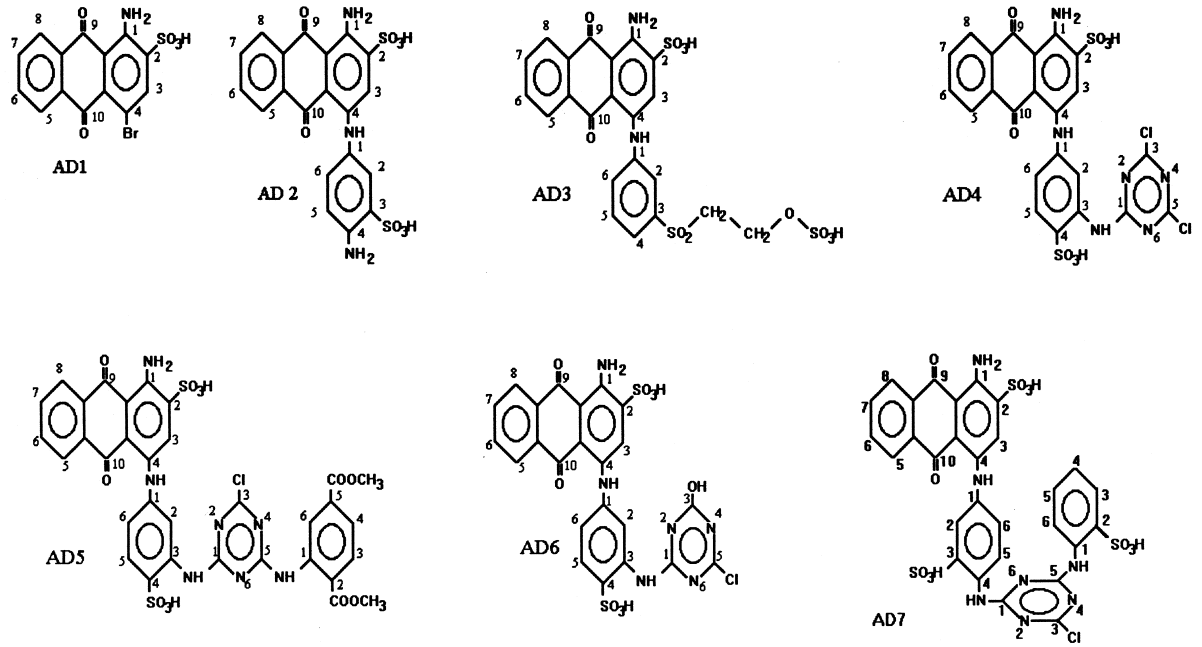

Fig. 1. The structures of the anthraquinone dyes. AD1: 1-amino-4-bromo-2-(9,10-anthraquinone)-sulfonic acid; AD2: 1-amino-4-(4-amino-3-sulfanilino)-2-(9,10-anthraquinone)-sulfonic acid; AD3: 1-amino-4-[4-(1-sulfonyl-ethyl-2-sulfoxy)]-2-(9,10-anthraquinone)-sulfonic acid; AD4: 1-amino-4-[3-(3,5-dichloro-2,4,6-triazinylamino)-4-sulfanilino]-2-(9,10-anthraquinone)-sulfonic acid; AD5: 1-amino-4-{3-[3-(2,5-dimethoxycarbonylanilino)-5-chloro-2,4,6-triazinylamino]-4-sulfanilino}-2-(9,10-anthraquinone)-sulfonic acid; AD6: 1-amino-4-[3-(3-chloro-5-hydroxy-2,4,6-triazynilamino)-4-sulfanilino]-2-(9,10-anthraquinone)-sulfonic acid; AD7: 1-amino-4-{4-[3-chloro-2,4,6-triazinyl-amino-5-(3-sulfanilino)-3-sulfanilino]}-2-(9,10-anthraquinone)-sulfonic acid. AD1–3 in contrast to AD4–7 do not contain the triazine moiety. AD3, 4 and 7 are commercially available and are classified as C.I. 19, C.I. 4 and C.1. 2.

receptors, AD7 may modulate the effect of ATP on cardiac contractility [14]; (ii) AD7 may mimic the ATP-mediated inhibition of the mitochondrial K^+ uniport [15]; (iii) as a nucleotide analogue [15], AD7 may antagonize the putative P2x [16] as well as the P2y [14] purine receptors; (iv) AD7 may also be applied as a pyrimidine nucleotide analogue that is not metabolized [17]. By means of model compounds with substructures corresponding to those of the blue dyes, it was revealed that the AD7-induced inhibition of both hexokinase and Na/K-ATPase involves the formation of irreversible adducts located either in the ATP-binding site of these enzymes or in its vicinity. The 1-amino-4(*N*-phenylamino)anthraquinone-2-sulfonic acid part [11] and the 1-amino-4(3-amino-4-sulfanilino)-2-(9,10-anthraquinone)-sulfonic acid part [9,12], were recognized as the minimal structures required for inhibitory interaction of AD7 with the molecules of hexokinase and Na/K-ATPase, respectively. Based on the above similarity in the minimal structures of the ligands, it may be assumed that a similarity may also exist in the reactivity of ATP-binding sites of the hexokinase and Na/K-ATPase. However, this may not concern all binding sites for nucleotides, since it has been reported that Remazol Brilliant Blue (AD3) acts as a strong inhibitor of Na/K-ATPase, but a weak inhibitor of LDH [9].

In our previous paper, we studied the inhibitory effect of eight AD derivatives on Na/K-ATPase activity [12]. In the present study, the inhibitory effect of the same AD derivatives on the activity of LDH is investigated. LDH was chosen since it is representative of enzymes utilizing NADH as a coenzyme. The present study focuses on quantitative structure–activity relationships and is based on the presumption that the interaction between ADs and the enzyme has an electrostatic character.

2. Experimental

2.1. Reagents and chemicals

Lactate dehydrogenase from rabbit skeletal muscle (LDH, EC 1.1.1.27), (lyophilized; specific activity: 140 μmol of pyruvate reduced by 1 mg of enzyme per minute, at 37°C and pH 7.0) was purchased from Fluka (Buchs, Switzerland). NADH, pyruvate, Cibacron Blue F3G-A (Reactive blue 2, AD7 in Fig. 1), further Remazol Brilliant Blue R (Reactive blue 19, AD3 in Fig. 1) and Procion Blue MX-R (Reactive Blue 4, AD4 in Fig. 1) were obtained from Sigma (St. Louis, MO, USA). The sodium salt of 1-amino-4-bromo-2-(9,10-anthraquinone)-sulfonic acid was prepared at the Research Institute of Organic Synthesis (AD1 in Fig. 1, Pardubice, Czech Republic). Other dyes (AD2, 5, 6 in Fig. 1) were prepared by ourselves as described previously [12]. Other substances were purchased from Serva (Heidelberg, Germany), Sigma and Lachema (Brno, Czech Republic). All commercially available chemicals were of analytical grade.

2.2. Estimation of LDH activity

The specific activity of LDH was determined by means of a diode-array spectrophotometer (Hewlett-Packard 8452 A) at λ=340 nm, ε=6700 l/mol·cm, directly in the photometric cuvette. Enzyme reaction was carried out for 3–5 min in a final volume of 3 ml at room temperature. The reaction medium contained (in m*M*): 50 phosphate buffer with 0.4 sodium bicarbonate (pH 6.5, 7.5 and 8.5); 0.73 pyruvate and 0.01–0.24 NADH in the presence or absence of 0.00125–0.02 of ADs as well as 20 μg of LDH. Initial velocities of the enzyme reaction were obtained from the linear part of the LDH-catalyzed NADH oxidation–time dependence curves.

2.3. Evaluation of enzyme kinetics

The kinetics of LDH activation by increasing concentrations of NADH was evaluated by means of the Michaelis and Menten relationship:

$$V_o = V_{max} c(c + K_m)^{-1} \quad (1)$$

where V and V_{max} represent the initial velocities of LDH reaction at NADH concentration c and $c \to \infty$, respectively, and K_m is the Michaelis-Menten constant.

Data obtained by direct measurement of inhibition of the LDH reaction by the ADs investigated were further processed by means of Eq. (2). The latter equation was derived from the Dixon's graphical method for evaluation of partially competitive inhibitors, i.e. classic Dixon transformation (the $1/V$ versus concentration of inhibitor i transformation) yielded concave curves [18].

$$V = V_o[1 + [i + (i/IC_{50})^n]^{-1} \quad (2)$$

where V and V_o represent the initial velocity of enzyme reactions at the concentration of inhibitor i and $i=0$, respectively. IC_{50} is the median inhibition concentration. The exponent n characterizes the concave shaped curvature of the relationship in the classic linear Dixon system, obtained for partial competitive inhibitors. In the case of competitive inhibitors, IC_{50} should depend linearly on the concentration of the substrate, i.e., NADH (c). The resulting lines intercept the ordinate and the intercepts are equal to K_i as follows from the equations of Dixon [18]:

$$IC_{50} = K_i + kc \quad (3)$$

Substitution of V_o and IC_{50} in Eq. (2), by Eqs. (1) and (3) gives Eq. (4). This expresses the initial velocity of the enzyme reaction as a function of two independent variables: the concentration of substrate and that of the tightly bound competitive inhibitor:

$$V = V_{max} c(c + K_m)^{-1}\{1 + [i/(K_i + kc)]^n\} \quad (4)$$

Eq. (4) was used for the estimation of the respective K_i values, utilizing the SigmaPlot Scientific graphic software, version 5.0 (Jandel Corporation 1986–1992).

2.4. Others methods

Three-dimensional structures of the respective ADs and NADH were computed using Desktop Molecular Modelling software (Oxford Electronic Publishing, Oxford, UK). The apparent acid-dissociation constants pK_a^{app}, reflecting the acid–basic

properties of single AD, were estimated by means of a titration, were already described previously [12]. Zonal chromatography of LDH was performed on cellulose modified by AD7 or AD3 (AD7-cellulose, AD3-cellulose) at 25±0.5°C. The size of the column was 29×1.1 cm I.D. and it was filled with the AD7- or AD3-cellulose equilibrated with different concentrations of NADH (20–500 m*M*) in a 20 m*M* phosphate buffer solution, pH 7.0. Preparation of both modified celluloses, their characterization, as well as all other details concerning the zonal chromatography procedure, were described previously [19,20].

3. Results and discussion

3.1. Characterization of anthraquinones

Structures of the anthraquinone dyes used in this study are shown in Fig. 1. Among them, the derivatives AD3, 4 and 7, i.e., Remazol Brilliant Blue R (Reactive blue 19), Procion Blue MX-R (Reactive Blue 4) and Cibacron Blue F3G-A (Reactive Blue 2), are also available commercially. The purity of derivatives was controlled by TLC [21,22] and always exceeded 95%. The structural analyses of the compounds synthesized have been described in the previous paper [12].

Among the compounds used in the present study, the anthraquinones 3–7 may be considered as reactive ADs, and four of them contain reactive chlorine located on triazine moieties (in position 3 on AD3, 4 and 7 as well as in position 5 on AD4 and 6). The reactive moiety of derivative AD3 represents an ethylenedisulfonate group. In comparison to these five derivatives, derivatives AD1 and AD2 may be considered as less reactive under physiological conditions. When applied, the acid–base behavior of anthraquinone derivatives does not follow an unambiguous titration curve, reflecting dissociated compartments that would inactivate the respective substituents due to the multiproton exchanging character of their molecules [12]. However, the acid–base properties of anthraquinones seem to be characterized by their apparent acid-dissociation constants, pK_a^{app}, that describe the net exchange of protons between the anthraquinone molecule and the surrounding aqueous solution (Table 1).

3.2. Effect of anthraquinone dyes on LDH activity

The LDH reaction was assessed spectrophotometrically by measuring the oxidation of NADH at 340 nm. Substances absorbing in the UV and VIS regions, such as anthraquinones (for example see AD7) did not interfer with the measurement (Fig. 2). Dependence of the initial velocity of LDH reaction

Table 1
Kinetic constants characterizing the inhibitory effect of ADs on LDH and apparent acid-dissociation constants

Anthraquinone dyes	pH	pK_a^{app}	*k*	*n*	K_i (μmol/l)
AD1	7.5	7.216	*	*	*
AD2	7.5	7.235	0.17±0.01	0.86±0.09	24.66±1.34
AD3	7.5	7.196	0.05±0.01	0.71±0.08	37.24±1.57
AD4	7.5	7.069	0.15±0.01	0.66±0.04	0.63±0.05
AD5	7.5	6.673	0.12±0.02	0.56±0.07	7.58±0.91
AD6	6.5	6.796	0.06±0.01	0.85±0.09	0.10±0.01
	7.5	6.796	0.05±0.01	0.45±0.03	0.51±0.02
	8.5	6.796	$0.01\pm0.00_1$	0.19±0.02	6.25±0.78
AD7	6.5	6.828	0.09±0.01	0.85±0.10	1.62±0.12
	7.5	6.828	0.08±0.02	0.87±0.09	2.71±0.18
	8.5	6.828	$0.01\pm0.00_1$	0.09±0.01	21.37±1.67

Michaelis-Menten equation [Eq. (1)] yields the following kinetic constants for NADH stimulation of LDH: at pH=6.5, V_{max}=44.95±3.95 μmol/mg·min and K_m=4.89±0.37 μmol/l; at pH=7.5, V_{max}=12.25±0.92 μmol/mg·min and K_m=13.01±1.12 μmol/l; at pH=8.5, V_{max}=1.08±0.08 μmol/mg/min and K_m=39.31±5.27 μmol/l. K_i, *k* and *n* were obtained by nonlinear regression of kinetic data according to Eq. (4) and are characterized by S_d value. pK_a^{app} were obtained by acid–base titration.
* AD1 did not exert any measurable inhibitory effect.

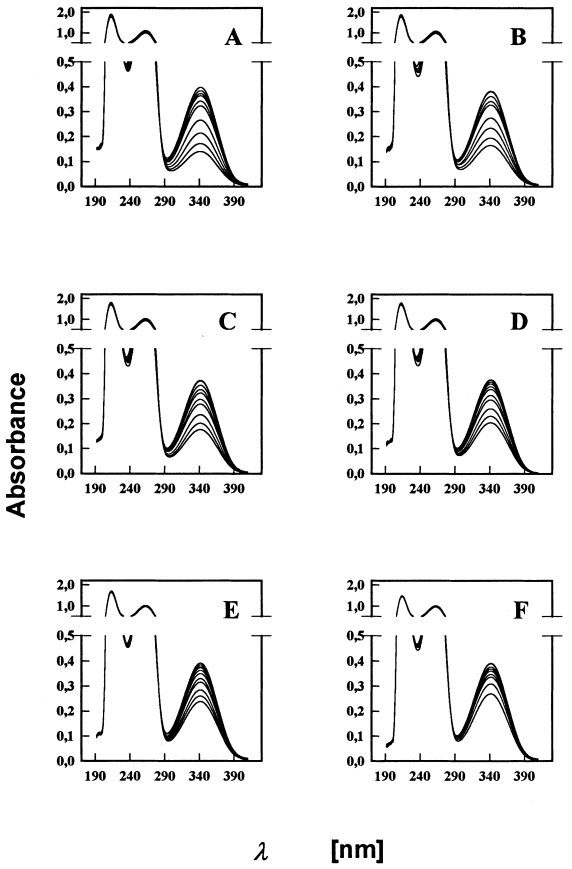

Fig. 2. The spectra of NADH oxidation by LDH in the absence (panel A) and the presence of AD7 at the following concentrations: 1.25 (panel B); 2.50 (panel C); 5.00 (panel D); 10.00 (panel E) and 20.00 (panel F) μ*M*. The spectra were registered at 0, 10, 20, 30, 45, 60, 90, 120, 180 and 240 s after the start of the LDH reaction by the addition of enzyme.

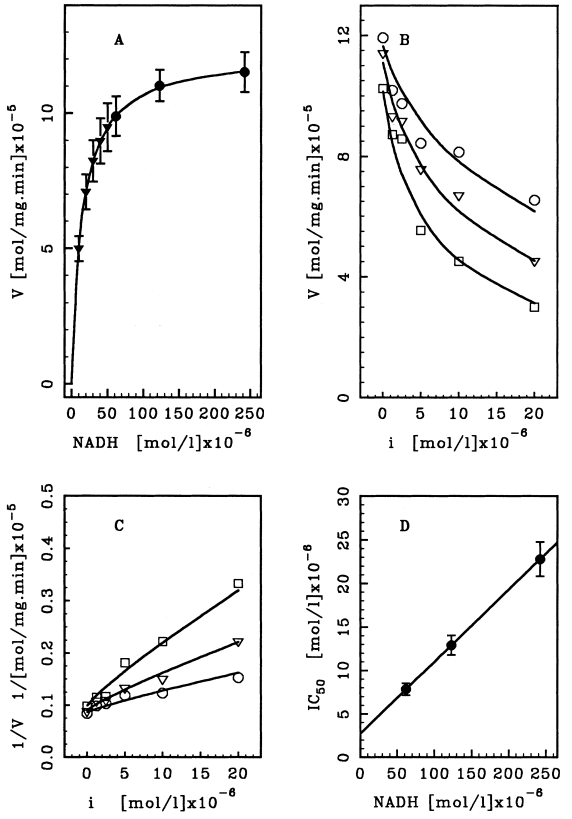

Fig. 3. Kinetic evaluation of AD7-induced inhibition of LDH activity. Panel A: The stimulation of LDH activity by NADH (as coenzyme). Panel B: The concentration dependence of the inhibitory action of AD7 on LDH activity registered at the various concentrations of NADH (□: 0.062 m*M*, ▽: 0.124 m*M*, ○: 0.248 m*M*). Panel C: Dixon's plots of AD7-induced inhibition obtained by transformation of the data from panel B to the coordination system 1/V vs. i. Panel D: Relationship between the IC_{50} and the NADH-concentration. Data represent means of three independent values; S.E.M. never exceed 3% of the mean.

on NADH concentration yields a classic hyperbolic curve that may be described by the Michaelis–Menten equation (Fig. 3A). When studying the inhibitory effect of the respective AD on LDH reaction, NADH concentrations of 62, 124 and 248 μ*M* (in Fig. 3A indicated by full circles) were used. With the exception of ADs 1, all ADs exerted an inhibitory influence on LDH reaction. Under experimental conditions applied, AD1 proved to be ineffective. Inhibitory influences of anthraquinone dyes on LDH reaction were fitted by Eq. (4) using the concentrations of both NADH, and the respective AD as two independent variables. Eq. (4) adequately describes the inhibitory influences of AD2-7 on the LDH reaction, as is demonstrated for AD7 in Fig. 3B. The kinetic variables (Table 1) applied in Eq. (4) were obtained by means of nonlinear regression. Since the inhibitory influences of anthraquinones AD2-7 on LDH activity could be described by Eq. (4), these substances may be considered as partial competitive inhibitors. In this case, when using the transformation according to Dixon [18], (Fig. 3C for AD7) slightly convex curves were obtained: the exponent $n<1$ (see Eq. (4)) was responsible for their curvature. The competition for the binding site on the LDH between AD7 and NADH is clearly docu-

mented by directly proportional dependence of IC_{50} values on NADH concentration (Fig. 3D). The values of IC_{50} were obtained by nonlinear regression of the single inhibition curves according to Eq. (2). Effects of the other anthraquinone dyes (AD2-6, not shown) on LDH activity were similar to those observed with AD7 (for the respective data see Table 1). Concerning Remazol Brilliant Blue (AD3) and Cibacron Blue F3GA (AD7), these were already found to inhibit competitively both the LDH-catalyzed reduction of NAD [9] and the oxidation of NADH [19], respectively.

Values of K_i characterizing the efficacy of anthraquinone dyes in the inhibition of LDH reaction are summarized in Table 1. AD4–7, that contain a reactive triazine part, exceeded in their inhibitory effect on LDH ($K_i < 10$ μM) the effects of AD2 and AD3 with triazine moiety absent (K_i values 24.66 and 37.64 μM, respectively). Concerning AD3 and AD7, these data confirmed our previous findings [9,19]. The study of equilibrium adsorption of LDH on porous bead cellulose derivatized by AD3 and AD7 revealed dissociation constants of 37.53 ± 0.02 μM and 0.46 ± 0.02 μM for the respective AD–LDH complexes [23]. The values of these dissociation constants agreed well with the respective K_i values given in Table 1. Our data also indicated that the K_i values seem to reflect predominantly the affinity of the ADs to LDH. In contrast to the stoichiometric interaction between LDH and the AD7 immobilized on bead cellulose, under similar conditions AD3 failed to exhibit stoichiometry [23,24].

Zonal affinity chromatography experiments with NADH as mobile ligand further revealed: (i) A concentration-dependent decrease in the elution volume of LDH on a column packed with AD7 attached to bead cellulose (Fig. 4A), that could be considered as a displacement phenomenon; (ii) complete lack of displacement phenomenon under these conditions when investigating AD3 (Fig. 4B). Hence, in the free state, AD3 and AD7 both exhibited NADH-mimicking properties (Table 1), whereas attached to bead cellulose, AD3 was found to loose its NADH-mimicking properties. It has been shown [25] that immobilization of the AD3 on bead cellulose involves the replacement of a sulfonic group located in the sulfoethylene side chain of the AD3 molecule by a glucosyl unit of cellulose. This may indicate that

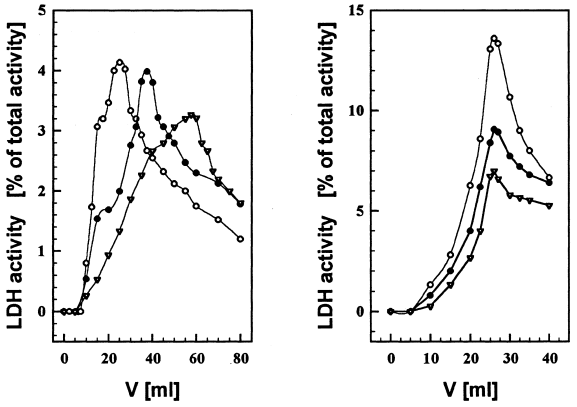

Fig. 4. Interaction of LDH with AD3 and AD7 linked with porous bead cellulose using zonal affinity chromatography. Panel A: Zonal chromatography of LDH on AD7-cellulose beads (138 mM/l of gel) using elution with mobile phase containing different concentrations of NADH (▽: 100 mM, ●: 300 mM, ○: 500 mM). Panel B: Zonal chromatography of LDH on AD3-cellulose beads (389 mM/l of gel) using elution with different concentrations of NADH (▽: 20 mM, ●: 50 mM, ○: 100 mM). Phosphate buffer (20 mM, pH 7.0) was used as a mobile phase in both cases. Data represent means of three independent values; S.E.M. never exceed 3% of the mean.

the above sulfonic group could play an essential role in the NADH mimicking properties of AD3. This idea is supported by AD1 that does not exhibit any sulfonic-group-containing side chain in respect to the anthraquinone moiety (Fig. 1) and, consequently, has no inhibitory effect on LDH. Conversely, AD2 and AD4–7 that contain side sulfobenzene moieties with free sulfonic groups (in position 3 on AD2 and in position 4 on AD4–7, Fig. 1) are all inhibitory to LDH. Moreover, while investigating AD7, we have found that this compound, that binds to bead cellulose via substitution of a reactive chlorine in the triazine part of its molecule, leaving its sulfonic-group-containing side chain free, preserves considerably its capability to mimic NADH, also in immobilized form. These findings lead to the conclusion that 1-amino-4-(*N*-phenylamino-(3 or 4)-sulfo-)-anthraquinone-2-sulfonic acid could represent the minimal structure required for inhibition of LDH activity. Similarly, in Ref. [11] it was recently identified that 1-amino-4-(*N*-phenylamino)anthraquinone-2-sulfonic acid is a minimal structure required for the inhibition of hexokinase activity. A similar minimal structure was also found to be

required for inhibition of the (Na/K)-ATPase [12]. This indicates that the above minimal structures exert essential influence on the NADH and/or ATP-mimicking properties of ADs. In this respect, the presence of a triazine moiety proved to considerably enhance the inhibitory effect of AD4-7 on LDH (Table 1), but exerted little influence on the inhibition of the (Na/K)-ATPase activity [9,12].

The porous bead cellulose, activated by AD3 or AD7 used in this paper for zonal affinity chromatography, represents an effective carrier for affinity chromatography used for purification of LDH [4,19,20,26,27] or glycerol kinase [8]. The replacement of NADH-dependent elution of bound LDH with the elution of enzymes by anthraquinone dye-linked dextran T10 in the previous papers [20,26,27] may represent an interesting possibility to decrease the expenses of enzyme purification using dye–ligand chromatography. Moreover, competitive elution of LDH with this dye-linked dextran [26,27] was found to be more effective as a common biomimetic with NADH.

It should be stressed that K_i values for inhibition of LDH by ADs show a tendency to be elevated with increasing pH of reaction mixtures, as shown for AD6 and AD7 in Table 1. While pH 6.5 is lower than the pK_a^{app} values for AD6 or AD7, both pH 7.5 and 8.5 exceed the pK_a^{app} values for both dyes. The change of pH from 7.5 to 8.5, i.e., to a pH value that exceeds the pK_a^{app} values for AD6 and AD7 by about more than 1.5 pH unit, induced an increase of K_i value of approximately one order (Table 1).

3.3. Relation between structures of dyes and their inhibitory effect on LDH activity

The above considerations concerning the crucial importance of the location of free sulfonic groups and that of the triazine moieties for inhibition of LDH by diverse ADs, as well as the assumptions that the interaction of ADs with LDH is of an electrostatic nature, were proposed to participate in the biorecognition of ADs by LDH. Accordingly, it may be assumed that similarities in mutual distances between the acidic and basic groups in molecules of NADH and those of the dyes mimicking NADH will determine the efficacy of their specific inhibitory action on LDH. Using the Desktop Molecular Modelling software (see Section 2.4) we accounted the three dimensional structures of each dye and the NADH.

A comparison of the latter structures revealed: (i) the distances (A') between the 1-NH_2-group of the anthraquinone part and the SO_3H-group of the diaminobenzene part of the ADs (AD2 in position 3, AD4-7 in position 4, Fig. 1), or the SO_3H-group of ethylenesulfone side chain of (AD3) mimic the distance ($A = 1.019$ nm) between the 6-NH_2-group of the adenine part and of the first phosphate group in the NADH molecule; (ii) the distances (B') between the nitrogen atom in the triazine part of the AD molecule (in position 2, Fig. 1) and the SO_3H-group in the anthraquinone part of the AD molecule (in position 2, Fig. 1) mimic the distance ($B = 0.709$ nm) between the nitrogen atom of the nicotinamide part and the second phosphate group of the NADH molecule (Table 2). The acid–base properties of ADs expressed by means of apparent acid-dissociation constant pK_a^{app} (see Section 2.4) may be considered as a further important factor that may influence their electrostatic interactions. Values of K_i obtained for ADs (Table 1) were fitted by using nonlinear regression according to Eq. (4). The positive value (a) of the expression $(A-A')/A'$, the positive value (b) of expression $(B-B')B'$ and the difference between the pH of the reaction medium and the pK_a^{app} value of ADs (c), were used as three independent variables.

Table 2
Distances A' and B' characterizing the anthraquinone dyes

Dyes	A' (nm)	B' (nm)
AD1	–	–
AD2	1.035	–
AD3	1.059	–
AD4	1.032	0.854
AD5	1.042	0.790
AD6	1.041	0.759
AD7	1.032	0.772

A': Distances between the 1-NH_2-group of the anthraquinone part and the SO_3H-group of the diaminobenzene part of the ADs (AD2 in position 3), AD4–7 (in position 4, Fig. 1) or the SO_3H-group of the ethylenesulfone side chain of (AD3). B': Distances between the nitrogen atom in the triazine part of the AD molecule (in position 2, Fig. 1) and the SO_3H group of the anthraquinone part of the AD molecule (in position 2, Fig. 1).

Table 3
Parameters of Eq. (4) obtained by nonlinear regression

Parameter	
P_1	0.819±0.067
P_2	4.849±0.710
P_3	1.417±0.126
P_4	0.302±0.120

Data represent computed values±S_d for 6 degrees of freedom. Values of K_i obtained for inhibition of LDH by ADs were fitted by nonlinear regression according to the following equation:

$$K_i = 10^{P_1 c + P_2 a + P_3 b + P_4}$$

a and *b* represents the positive values of expressions $(A-A')/A'$ and $(B-B')/B'$; *c* represents the difference between the pH of the reaction medium and the pK_a^{app} value of ADs; Values of A' and B' were shown in Table 2; *A* represents the distance between the 6-NH_2 group of the adenine part and of the first phosphate group in the NADH molecule, *B* represents the distance between the nitrogen atom of the nicotinamide part and the second phosphate group of the NADH molecule.

$$K_i = 10^{P_1 c + P_2 a + P_3 b + P_4}$$

where P_1–P_4 are regression coefficients obtained by nonlinear regression summarized in Table 3.

Comparing the K_i values computed according to Eq. (4) (using parameters P_1–P_4) with the corresponding K_i values obtained by measuring enzyme kinetics (Table 1) good agreement was obtained (Fig. 5). This indicated that the independent variables (*a*, *b*, *c*) may represent important structural determinants of ADs that could at least be partially responsible for their capability to inhibit LDH. It also follows from Eq. (4) that if parameters P_1–$P_3 > 0$, the increase in respective independent variables will be accompanied by an increase in the K_i value i.e., with a decrease in the inhibitory effect of ADs. The above relationship also indicates that the increasing difference in distances *A*:*A*′ and *B*:*B*′ will predict a decrease in the inhibitory effect of the respective AD. The meaning of the described negative effect of variable *c* on LDH inhibition by ADs indicated that effective inhibition of LDH may be achieved when the pK_a^{app} value of ADs exceeds the pH value of the reaction medium. Hence, it seems that ADs interact with LDH in the protonated form.

In conclusion, it should be stressed that the relationships between the structure of ADs and their inhibitory effect on LDH described in the present study could be useful for designing dye structures that enable them to interact with LDH more specifically. Comparison of the presented results with results of other papers studying the relationships between the structure of ADs and their inhibitory effect on other enzymes like ATPases [12], dehydrogenases [13] and oxidoreductases [28] could enable the deduction of the proper structure of ADs that may ensure better selectivity in interaction with a given enzyme.

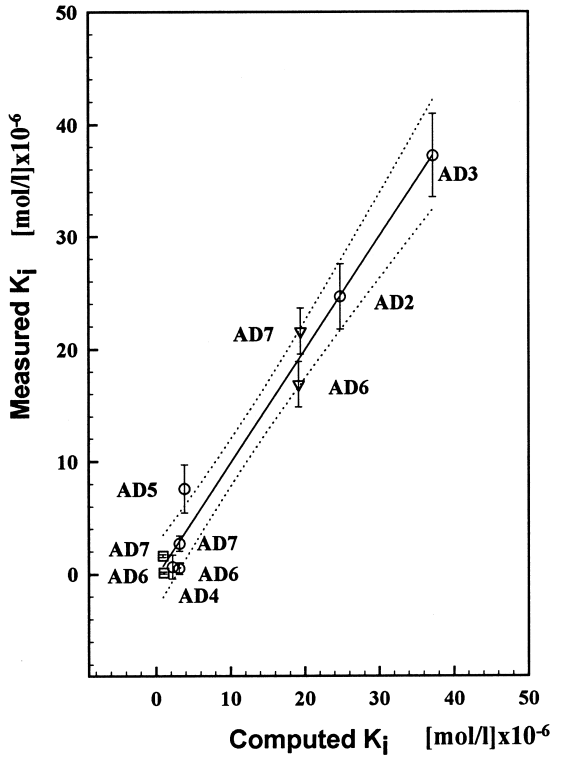

Fig. 5. Correlation between measured and computed inhibition constants (K_i) for the effect of ADs on the LDH activity at the various pH (□: 6.5, ○: 7.5, ▽: 8.5). K_i values were obtained as parameters of Eq. (4) by nonlinear regression using *a*, *b* and *c* (see Section 3.3) as three independent variables.

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References

[1] S.T. Thompson, K.H. Cass, E. Stellwagen, Proc. Natl. Acad. Sci. USA 72 (1975) 669–672.
[2] S. Subramanian, Crit. Rev. Biochem. 16 (1984) 169–205.
[3] K.D. Kulbe, R. Schuer, Anal. Biochem. 93 (1979) 46–51.
[4] P. Gemeiner, D. Mislovičová, J. Zemek, L. Kuniak, Collect. Czech. Chem. Commun. 46 (1981) 419–427.
[5] G. Kopperschäger, H.J. Böhme, E. Hofmann, in: A. Fiechter (Ed.), Advances in Biochemical Engineering, Springer-Verlag, Berlin, 1982, pp. 101–138.
[6] M.D. Scawen, J. Darbyshire, M.J. Harvey, T. Atkinson, J. Biochem. 203 (1982) 699–705.
[7] Y. Kroviarski, S. Cochet, C. Vadon, A. Truskolaski, P. Boivin, O. Bertrand, J. Chromatogr. 499 (1988) 413–422.
[8] V. Žúbor, A. Breier, M. Horváthová, D. Hagarová, P. Gemeiner, D. Mislovičová, Collect. Czech. Chem. Commun. 58 (1993) 445–451.
[9] V. Ďurišová, A. Vrbanová, A. Ziegelhöffer, A. Breier, Gen. Physiol. Biophys. 9 (1990) 519–528.
[10] T. Higichi, Y. Imamura, M. Otagiri, Biochim. Biophys. Acta 1158 (1993) 23–28.
[11] R.N. Puri, R. Roskoski, Biochem. J. 300 (1994) 91–97.
[12] P. Dočolomanský, V. Boháčová, A. Breier, Chem. Papers 48 (1994) 128–132.
[13] N.E. Labrou, Y.D. Clonis, Arch. Biochem. Biophys. 316 (1995) 169–178.
[14] L. Mantelli, S. Amerini, S. Filippi, F. Ledda, J. Pharmacol. 104 (1993) 1268–1271.
[15] A.D. Beavis, K.D. Garlid, J. Biol. Chem. 268 (1993) 997–1004.
[16] A.D. Michel, P.P. Humphrey, Arch. Pharmacol. 348 (1993) 608–617.
[17] P.V. Rao, J. Horwitz, J.S. Zigler, J. Biol. Chem. 269 (1994) 13266–13272.
[18] M. Dixon, Biochem. J. 127 (1972) 197–202.
[19] D. Mislovičová, P. Gemeiner, A. Breier, Enzyme Microb. Technol. 10 (1988) 568–573.
[20] D. Mislovičová, P. Gemeiner, V. Ďurišová, Collect. Czech. Chem. Commun. 55 (1990) 581–586.
[21] R.S. Biessner, F.B. Rudolph, Arch. Biochem. Biophys. 189 (1978) 76.
[22] M. Federici, E.R. Stadtman, Biochemistry 24 (1985) 647–660.
[23] A. Breier, V. Ďurišová, T. Stankovičová, D. Mislovičová, P. Gemeiner, J. Biochem. Biophys. Methods 22 (1991) 185–193.
[24] A. Breier, P. Gemeiner, A. Ziegelhöffer, L. Turi-Nagy, V. Štofaníková, Colloid. Polym. Sci. 265 (1987) 933–937.
[25] P. Gemeiner, M.J. Beneš, J. Štamberg, Chem. Papers 43 (1989) 805–848.
[26] D. Mislovičová, P. Gemeiner, E. Stratilová, M. Horváthová, J. Chromatogr. 510 (1990) 197–204.
[27] P. Gemeiner, M. Polakovič, D. Mislovičová, V. Štefuca, J. Chromatogr. B, this issue. (in press AF2O=Y449).
[28] T. Prestera, H.J. Prochaska, P. Talalay, Biochemistry 31 (1992) 824–833.