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Review

Biomimetic dyes as affinity chromatography tools in enzyme purification

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Abstract

Affinity adsorbents based on immobilized triazine dyes offer important advantages circumventing many of the problems associated with biological ligands. The main drawback of dyes is their moderate selectivity for proteins. Rational attempts to tackle this problem are realized through the *biomimetic dye* concept according to which new dyes, the *biomimetic dyes*, are designed to mimic natural ligands. Biomimetic dyes are expected to exhibit increased affinity and purifying ability for the targeted proteins. Biocomputing offers a powerful approach to biomimetic ligand design. The successful exploitation of contemporary computational techniques in molecular design requires the knowledge of the three-dimensional structure of the target protein, or at least, the amino acid sequence of the target protein and the three-dimensional structure of a highly homologous protein. From such information one can then design, on a graphics workstation, the model of the protein and also a number of suitable synthetic ligands which mimic natural biological ligands of the protein. There are several examples of enzyme purifications (trypsin, urokinase, kallikrein, alkaline phosphatase, malate dehydrogenase, formate dehydrogenase, oxaloacetate decarboxylase and lactate dehydrogenase) where synthetic biomimetic dyes have been used successfully as affinity chromatography tools.

Keywords: Reviews; Affinity chromatography; Dyes; Biomimetic dyes; Enzymes

Contents

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1. Biomimetic dyes: necessity and chance

Immobilized textile triazine dyes, particularly Cibacron blue 3GA or F3GA (CB3GA), have been used as affinity chromatography tools for protein and enzyme purification for about 30 years. The originally employed dyes were commercial textile chlorotriazine polysulfonated molecules which are readily immobilized on polymers bearing hydroxyl groups. The low cost of these dyes, their ease of immobilization and resistance to biological and chemical degradation, and the high protein-binding capacity of the corresponding adsorbents, has led to affinity chromatography materials that are much less expensive and more stable than those based on natural biological ligands. Although textile dyes, in some case, interact with proteins with remarkable degrees of specificity [1–3], their interaction with a large number of seemingly unrelated proteins inevitably compromises their protein binding specificity and endow these molecules with a serious drawback. One way to cope with lack of specificity of immobilized dyes is to use specific eluents which allow to elute specifically the target protein with minimal contamination. This approach is better known as *affinity elution*, and it has been used in numerous affinity purifications with immobilized dye-ligands, including works of the present research group. If the dye interacts with the active site of the target enzyme, then an appropriate competing substance (ligand of the enzyme) can be used as a specific eluent of the enzyme. If, however, there is uncertainty on the mode of interaction between dye and enzyme, many substances can be tried as candidate eluting agents, with the hope that one will act as a selective competitor, leading to specific elution of the enzyme. Another strategy which can cope with the drawback of textile dyes, is to design new dye-ligands of improved affinity and specificity for the target protein. This is the subject of this review. In principle this can be achieved by designing synthetic dyes which mimic the structure and binding of natural biological ligands of the targeted protein. This new type of ligand is termed *biomimetic dye-ligand*, and it should not only display high specificity for the target protein, but also retain most of the advantages of the commercial textile dyes (termed *parent*, *nonbiomimetic* or *conventional* dyes).

However, conceiving an idea is not always enough. For its materialization often the right people must be at the right place and time. Although early in the 1980s the drawback of textile dyes had become clear, little could be done about it apparently due to the lack of expertise on dye synthesis and of the inaccessibility to certain dye ‘‘building blocks’’ by most academic institutions involved in this scientific area. It was good fortune, a chance for dye biotechnology, that Dr. C.V. Stead, an expert on textile dye synthesis at a major dye manufacturer, became involved at the right time with the research group of Lowe and co-workers (Cambridge, UK). This collaboration led to the materialization of the biomimetic dye concept for protein purification, and the ‘‘birth’’ of the first biomimetic cationic yellow dye [4] and the first biomimetic anionic blue dye [5].

2. First generation biomimetic dyes

Early in the 1980s, the time the first biomimetic dye was under design, development and assessment, were not available advanced molecular modeling software packages for application on a personal workstation, and for the sophisticated type of work required for ligand design and the study of ligand–enzyme interaction. Therefore, biomimetic dye design was relying on the known binding preference of the target enzyme for natural ligands, X-ray crystallography data, and other available useful biochemical information.

2.1. A biomimetic ligand for trypsin

The design of the first biomimetic dye was based

on the uncommon binding preference for cationic substrates of some enzymes of commercial and physiological significance, the trypsin-like proteolytic enzymes. They possess similar catalytic mechanism and bind the cationic side chains of Lys and Arg of their natural peptide substrates in a primary binding pocket proximal to the reactive Ser. Furthermore, the anionic carboxylate of an Asp, located at the bottom of the binding site, is important for the peptide–enzyme interaction. Benzamidine, a potent trypsin inhibitor, mimics the side chain of Arg in that it possesses both a cationic amidine group and a hydrophobic phenyl ring of comparable size to the recognizable group of the amino acid [6–8].

Therefore, the first biomimetic dye (Fig. 1a) was designed on the basis of the above information, by simply linking benzamidine to the reactive chlorotriazine ring via a diaminomethylbenzene group. The immobilized form of this biomimetic cationic yellow dye binds trypsin, thrombin, carboxypeptidase B [4] and urokinase [9] but neither the serine protease chymotrypsin, which prefers substrates with hydrophobic amino acids for its primary binding pocket, nor the unrelated proteins albumin and lactate dehydrogenase. In fact this biomimetic affinity adsorbent is able to completely separate the activity of trypsin (11-fold purification, 79% recovery) from chymotrypsin from bovine pancreatic extract [4]. This work was accomplished in 1984, but it was not published until 1987 because of considerations for a patent application.

2.2. Biomimetic ligands for kallikrein

In a pancreatic extract, besides trypsin and chymotrypsin, one other important proteolytic activity present is that of kallikrein. While trypsin is adsorbed on the immobilized mono-cationic dye of Fig. 1a, kallikrein is not. X-Ray crystallographic studies have shown that benzamidine binds in the primary binding pocket of pancreatic trypsin and forms a salt bridge with the negative side chain of Asp-189 at the bottom of the pocket. The amidinium nitrogens position symmetrically opposite the carboxylate oxygens of Asp-189 while the phenyl ring is sandwiched between the main chain atoms of residues 190–191 and 215–216 [6,10]. In porcine pancreatic kallikrein, the phenyl ring of the benzamidine is sandwiched similarly as in trypsin, but replacement of Gly-226 at the back of the pocket in trypsin with a bulkier Ser in kallikrein prevents the amidine group of benzamidine from symmetrically opposing the carboxylate of Asp-189, rotating it by 60°. As a result, only one hydrogen bond is formed between the amidinium nitrogens and the carboxylate oxygens of Asp-189 [11]. This is probably the reason for a 32-fold poorer affinity of benzamidine for kallikrein than for trypsin.

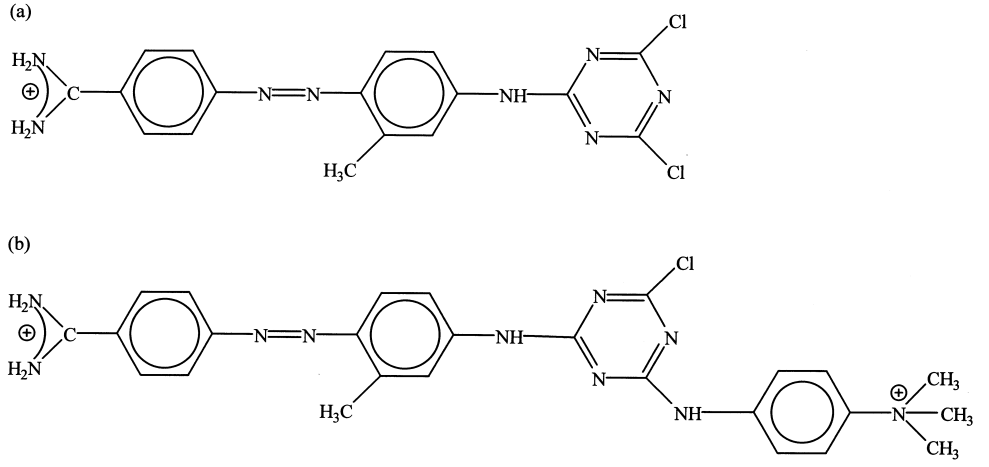


Fig. 1. Structures of the benzamidino-biomimetic triazine dyes, (a) mono-cationic and (b) bis-cationic.

It was, therefore, necessary to design a new dye-ligand that would bind kallikrein but neither trypsin nor chymotrypsin. For this purpose were synthesized and assessed dye-ligands bearing two recognition moieties on the triazine ring. Two of these ligands were bis-cationic, bearing an amino-benzamidine group and either an aminophenyl-trimethylammonium group (Fig. 1b) or an amino-benzamidine group. Both ligands displayed the highest affinity for kallikrein [12]. The immobilized form of the bis-cationic ligand of Fig. 1b is able to bind and purify kallikrein by 9.5-fold from a crude pancreatic extract in 79% yield, and generate a product 99.9% free of contaminating trypsin activity [12]. The same affinity adsorbent does not bind chymotrypsin.

Because kallikrein prefers ligands which bind in the primary and secondary substrate binding sites with cationic and hydrophobic groups, respectively, the enhanced inhibitory/binding behavior of the bis-cationic ligands designed for kallikrein is likely to be due to the presence of a second binding site on the protein surface at which the additional cationic group can bind [12].

2.3. A biomimetic ligand for alkaline phosphatase

A fine example of a purpose-designed biomimetic dye-ligand is that of the anthraquinone terminal-ring *p*-aminobenzyl phosphonic acid analogue (Fig. 2a) of the well known textile dye Cibacron blue 3GA or F3GA (Fig. 2b). The targeted enzyme is alkaline phosphatase from calf intestinal mucosa, which is widely used in immunodiagnostics and molecular biology. This enzyme is a orthophosphate monoester phosphohydrolase and it binds on immobilized CB3GA and phosphonate derivatives. Lindner et al. [5], without making use of bioinformatics (e.g., X-ray crystallography data and molecular modeling), simply replaced the terminal aminobenzene-sulfonate ring of CB3GA for a substrate analogue of alkaline phosphatase, *p*-aminobenzyl-phosphonate ring. The respective new blue biomimetic dye, when immobilized on agarose, is able to purify alkaline phosphatase by 280–330-fold in one chromatography step from calf intestinal extract, after specific elution with inorganic phosphate (5 m*M*).

The same biomimetic phosphonated blue ligand, when immobilized via a diaminohexane spacer molecule on high-performance chromatography synthetic support, is able to purify alkaline phosphatase from the same source by 120–140-fold [9].

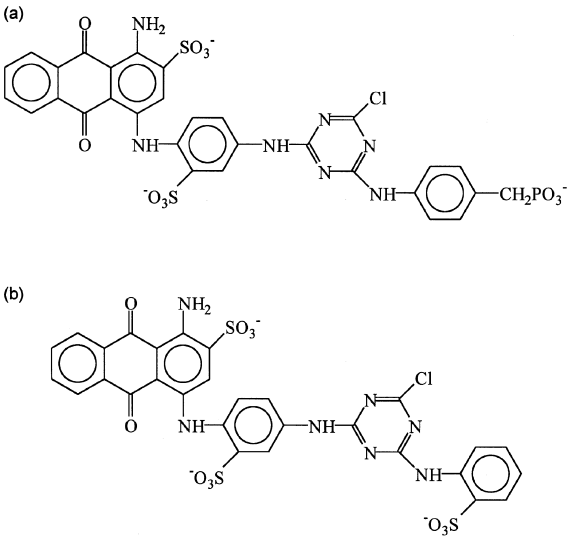


Fig. 2. Structures of the anthraquinone triazine dyes, (a) phosphono-biomimetic and (b) Cibacron blue 3GA.

For both cases above, the respective ''control'' CB3GA adsorbents purify the enzyme by approximately 20-fold [5,9] after biospecific elution with inorganic phosphate. An optimized purification procedure [13] led to 290- and 128-fold purification after biospecific elution with α-naphthyl phosphate and inorganic phosphate, respectively, from a CB3GA-Sepharose column.

2.4. Designed dye-ligands for alcohol dehydrogenase

Alcohol dehydrogenase from horse liver was yet another target for dye-ligand design [14,15]. This work was based on information from X-ray crystallographic studies which were performed by Biellman and co-workers in 1979. The synthesized and assessed dye-ligands were *o*-, *m*- and *p*-terminal ring analogues of CB3GA bearing sulfonate, carboxylate, phosphonate, alcoholic, amido and trimethylammonium groups as terminal-ring substituents [14]. It was reported that the differences in affinity for alcohol

dehydrogenase displayed by the dye-ligands in free solution were not translated into an analogous chromatographic behavior after the ligands were immobilized on agarose. Surprisingly, it was not a designed biomimetic dye-ligand, mimicking natural ligands of the enzyme, that has shown the highest binding and purifying ability. In fact the highest purification (10.3-fold) was shown by CB3GA substituted with a methoxy group at its triazine ring and immobilized from its anthraquinone moiety via a spacer molecule [15].

3. Second generation biomimetic dyes: design by computer aided molecular modeling and use of bioinformatics

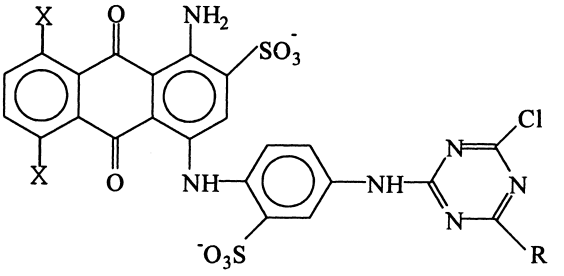
X-Ray crystallography data and the knowledge of the three-dimensional co-ordinates are very useful when one attempts to design a ligand for the binding site of a macromolecule. In principle, one can crystallize and study the ligand–protein complex, but this is neither always possible nor an easy task. The three-dimensional structural information became easier exploitable for routine ligand design after the introduction of advanced software packages for molecular graphics (e.g., Quanta, Insight) which can be used on a desktop graphics workstation (e.g., Silicon Graphics Iris, O2 or Octane). In fact this computational technology, an area of bioinformatics, has marked a new era in ligand design, offering the possibility of ''virtual reality'' for many ligand–protein complexes. However, when employing molecular graphics for effective ligand design one should take into consideration not only the feasibility and ease of chemical synthesis but also the steric shape, the distribution and linkage of functional groups.

The first applications of contemporary molecular modeling and bioinformatics to the design of biomimetic dye-ligands targeted two enzymes of analytical interest, L-malate dehydrogenase (MDH) [16,17] and L-lactate dehydrogenase [18] from bovine heart. The new biomimetic dye-ligands, designed for the above enzymes, also find application to the purification of other enzymes whose substrates share common features.

3.1. Biomimetic ligands for malate dehydrogenase

Fig. 3 shows the structures of the new biomimetic dye-ligands (BM) and the respective experimental K_D values for bovine heart MDH. Each biomimetic dye (for the synthetic procedures see Ref. [19]) is composed of two enzyme-recognition moieties, and it is therefore a chimeric molecule. The terminal biomimetic moiety (R–) bears a carboxyl or a ketoacid structure linked to the triazine ring, thus mimicking natural ligands (substrates, inhibitors) of MDH. The chromophore anthraquinone moiety remains unchanged and the same as that of the parent dichloro dye (VBAR), recognizing the nucleotide-binding site of MDH.

Molecular modeling and kinetic inhibition studies, as well as K_D determinations by both difference



Dye-ligand (-R)	K_D (μM)	
	by inactivation (pH 8.5)	by difference spectra (pH 7.5)
(1) -*m*-HN-C_6H_4-COO^-	6.6 ± 0.5	7.1 ± 0.2
(2) -*m*-HN-C_6H_4-CH_2COO^-	3.7 ± 0.04	5.5 ± 0.4
(3) -$HNCH_2COO^-$	20.3 ± 1.60	17.1 ± 0.8
(4) -$HN(CH_2)_2COO$-	24.9 ± 1.80	25.7 ± 1.9
(5) -*p*-HN-C_6H_4-$NHCOCOO^-$	2.0 ± 0.05	1.3 ± 0.03
(6) -SCH_2COCOO^-	6.9 0.70	18.3 ± 1.5
(7) -$HN(CH_2)_2NHCOCOO^-$	3.0 ± 0.2	1.8 ± 0.03
(8) -*o*-HN-C_6H_4-SO_3^- (CB3GA)	4.5 ± 0.30	5.9 ± 0.5
(9) -$O(CH_2)_2N^+(CH_3)_3$ (VBARCh)	-	285 ± 15
(10) -Cl (VBAR)	14.4 ± 1.3	-
(11) -OH (BMX3G)	-	245 ± 16

Fig. 3. Binding characteristics of biomimetic dyes BM (1–7), parent dyes Vilafix blue A-R (VBAR) and Cibacron blue 3GA (CB3GA), and control dyes choline-VBAR (VBARCh) and Procion blue MX-3G (BMX-3G) to bovine heart mitochondrial L-malate dehydrogenase. For all dyes X=H, except for BMX3G where one X=SO_3^- and the other X=H. (Data were obtained from Ref. [17]).

spectra and enzyme-inactivation studies, were used to assess the ability of the chimeric biomimetic dyes to act as affinity ligands for MDH. The BM dyes did not inactivate MDH but competitively inhibited inactivation by the dichloro dye VBAR which is known to react with the coenzyme binding site of MDH [16]. When compared to VBAR, Cibacron blue 3GA and two control non-biomimetic dyes (Nos. 9 and 11), two biomimetic dyes which were predicted by molecular modeling as being the most satisfactory (BM5 and BM7), exhibited the lower experimental K_D values and therefore the higher affinity for MDH. The biomimetic dye bearing *p*-aminophenyloxanilic acid as its terminal biomimetic moiety (BM5) exhibited the highest affinity (K_D 1.3 μM), and displayed competitive inhibition with respect to both NADH and oxaloacetate. The construction of the stereodiagram in Fig. 4 is based on the use of bioinformatics. It shows the biomimetic dye in the coenzyme-binding site of pig mitochondrial MDH. The effective accommodation of BM5 in the binding site of MDH is explained bellow.

The anthraquinone moiety is placed in the position of the adenine of NAD^+ in a hydrophobic crevice of MDH. The bottom of the cleft is blocked by Tyr-32 and Leu-100 so the anthraquinone cannot enter deeper into the crevice. This results in the exposure of the sulfonate, amino group and one of the carbonyl groups of the ring, all pointing towards the solvent, while the carbonyl group on the other side of the anthraquinone is placed within hydrogen-bonding distance of Asp-33. The easy accessibility of the amino group of the anthraquinone leads to the speculation that immobilization of BM5 on a solid support via this position should lead to an effective affinity adsorbent.

The triazine ring binds in the region where the coenzyme pyrophosphate binds, with the one side

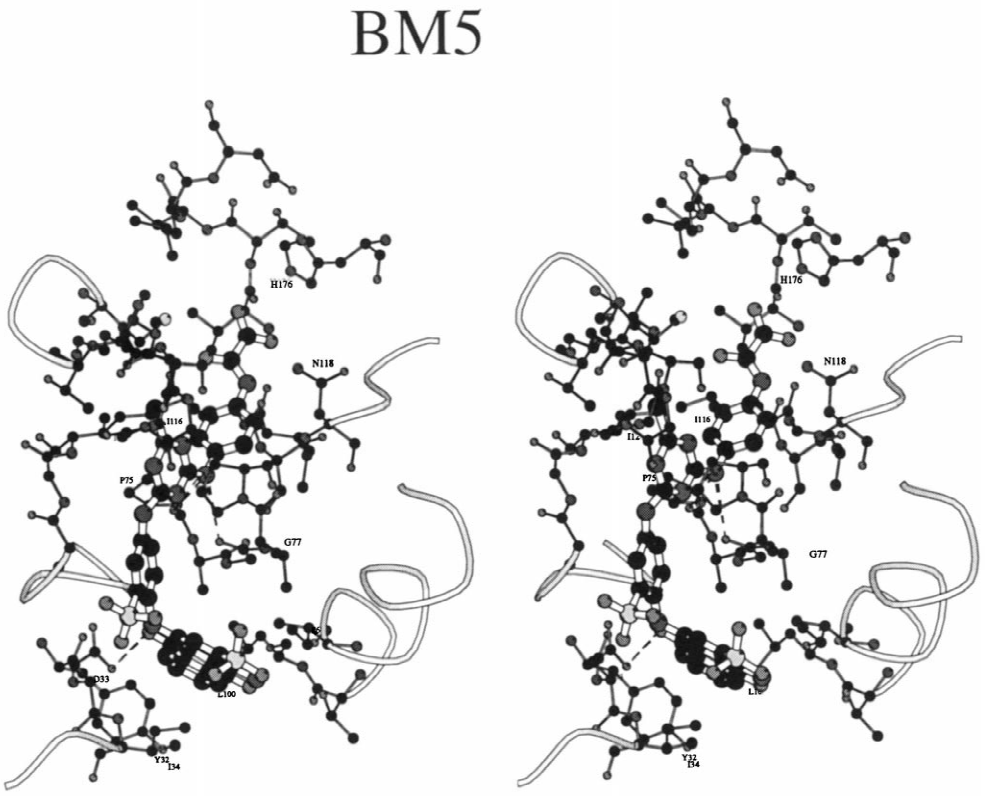


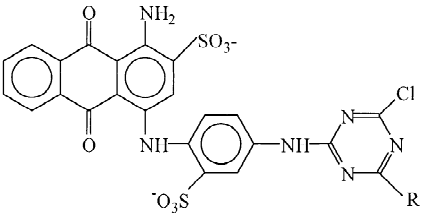
Fig. 4. Stereodiagram of the ketocarboxyl-biomimetic dye BM5 in the coenzyme-binding site of pig mitochondrial MDH (obtained from Ref. [17]).

close to the surface of MDH. This is a second position for effective immobilization of BM5 on a solid support probably without drastically altering its binding to MDH. On the other side of the triazine ring, towards the inner of the binding site, the biomimetic moiety is linked to the triazine ring via an amino group positioned within a hydrogen bond distance from the carbonyl of Gly-77.

With regards to the biomimetic moiety of ligand BM5, the benzene ring is placed in the space occupied by the second ribose ring (linked to the nicotinamide) of NAD^+ in the hydrophobic cavity formed by Ile-12, Ile-116, Asn-118, Pro-75 and fragments of the main chain of MDH. The use of a benzene ring keeps the direction of the ligand towards the catalytic site and provides spacing without introducing extra degrees of freedom. With regards to ligand BM7, the ethyl group occupies the same cavity but it is shorter in length, thus the ligand is less effective. The terminal keto-carboxylate group is attached to the benzene (BM5) or ethyl (BM7) group via an amide group as a spacer. In the longer of the biomimetic analogues (BM5) the keto-carboxylate superimposes readily with the amide group of the nicotinamide moiety of NAD^+ whereas the other analogues (Fig. 3) are shorter.

The differences in K_D values for MDH displayed by the biomimetic dye-ligands in free solution (Fig. 3) were not translated into an exactly analogous chromatographic behavior when using the immobilized forms of the ligands (Fig. 5). However, the most promising biomimetic ligand (BM5), as assessed from free solution studies and predicted by bioinformatics, also produced the most effective affinity adsorbent for the purification of MDH from bovine heart extract (Fig. 5, 5.7-fold purification) [20]. After optimizing the purification procedure, mitochondrial MDH was purified in two chromatography steps from bovine heart extract. The purified product showed a specific activity equal to 1300 U/mg and a single band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis [20].

The same biomimetic affinity adsorbent was also used for the rapid purification of a new MDH from *Pseudomonas stutzeri* [21]. This method employed only two chromatography steps (Table 1) and led to homogeneous enzyme, as judged from the single



Dye analogue (-R)	SA (U/mg)		Purification (fold)		Recovery (%)	
	KCl	NADH	KCl	NADH	KCl	NADH
BM1. -*m*-HN-C_6H_4-COO^-	273	337	1.8	2.8	98	82
BM2. -*m*-HN-C_6H_4-CH_2COO^-	364	539	2.4	4.5	100	91
BM3. -$HNCH_2COO^-$	280	377	1.8	3.1	98	80
BM4. -$HN(CH_2)_2COO^-$	370	511	2.4	4.2	100	91
BM5. -*p*-HN-C_6H_4-$NHCOCOO^-$	526	692	3.5	5.7	96	91
BM6. -SCH_2COCOO^-	302	502	2.0	4.1	91	89
BM7. -$HN(CH_2)_2NHCOCOO^-$	325	413	2.1	3.4	100	83
8. -*o*-HN*Bez*SO_3^- (CB3GA)	224	391	1.5	3.2	94	91
9. -Cl (VBAR)	300	388	2.0	3.2	96	90

Fig. 5. Evaluation of affinity dye-adsorbents for binding MDH activity from bovine heart extract. (Data were obtained from Ref. [20]). Procedures were performed at 4°C. Immobilized dye concentration: 2.2±0.2 μmol dye/g wet gel. Equilibration buffer: 20 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH, pH 7.0, containing 0.2% (v/v) β-MeSH. Bound proteins were eluted with 1 *M* KCl or 5 m*M* NADH. SA=specific activity

Table 1
Summary of purification procedure of malate dehydrogenase from *Pseudomonas stutzeri* (data were obtained from Ref. [21])

Step	SA[a] (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	30	1.0	100
DEAE-Sepharose ion-exchange chromatography, 6 ml gel, (KCl elution)	915	30.5	100
Biomimetic-dye affinity chromatography, 3 ml gel, (NADH elution)	2460	82.0	48

[a] SA=Specific activity.

band obtained after SDS–PAGE analysis and staining both with Coomassie blue and silver nitrate.

3.2. Biomimetic ligands for (keto)carboxyl-group recognizing enzymes

The (keto)carboxyl-biomimetic dye structures shown in Fig. 3, are effective affinity ligands for several carboxyl- and ketocarboxyl-group recognizing enzymes. In their immobilized form, these ligands can be used as affinity chromatography tools for the purification of several such enzymes (Table 2).

Formate dehydrogenase is purified from *Candida boidinii* (specific activity, SA>7 U/mg, recovery> 60%) in one affinity chromatography step on the mercaptopyruvic-biomimetic dye-ligand (BM6) [24], oxaloacetate decarboxylase is purified from *Pseudomonas stutzeri* (SA>300 U/mg, recovery>30%) in three chromatography steps on the *m*-aminobenzoic-biomimetic dye-ligand (BM1) [27], and oxalate oxidase is purified from barley seedlings (SA>30 U/mg, recovery>40%) in two chromatography steps on the aminoethyloxamic-biomimetic dye-ligand (BM7) [28].

3.3. Biomimetic ligands for lactate dehydrogenase

The purification of bovine heart lactate dehydrogenase (LDH) has also been studied with ketocarboxyl-biomimetic structures of Fig. 3 [22,23]. Three such biomimetic dye-ligands bearing as a triazine-linked terminal moiety a ketocarboxylate structure, which mimics natural substrates and inhibitors of LDH, were immobilized on agarose. The corresponding biomimetic adsorbents, along with non-biomimetic adsorbents bearing CB3GA and VBAR, were evaluated for their ability to purify LDH from bovine heart crude extract. When compared to non-biomimetic adsorbents, all biomimetic adsorbents exhibited higher purifying ability. Further, adsorbents bearing mercaptopyruvic acid (BM6) and *p*-aminophenyloxanilic acid (BM5) as biomimetic moiety, displayed the purifying ability (10- and 9.1-fold, respectively). These biomimetic adsorbents are effective for the purification of LDH from bovine heart in a two-step procedure: a biomimetic-dye chromatog-

Table 2
Enzymes purified and/or studied with (keto)carboxyl-biomimetic dye ligands

Enzyme	Source	Ref.
Formate dehydrogenase	*Candida boidinii*	[19,24]
Lactate dehydrogenase	Bovine heart	[18,22,23]
Lactate dehydrogenase	*Pagellus erithrinus*	[29]
Malate dehydrogenase	Bovine heart	[16,17,20,23]
Malate dehydrogenase	*Pseudomonas stutzeri*	[21]
Oxalate decarboxylase	*Aspergillus pheonicis*	[25]
Oxalate oxidase	*Hordeum vulgare* (barley)	[25,28]
Oxaloacetate decarboxylase	*Pseudomonas stutzeri*	[26,27]

raphy (elution with 0.1 *M* NAD^++1 m*M* sulfite), followed by DEAE anion-exchange chromatography (elution with KCl). The purified enzyme exhibits a specific activity equal to approximately 480 U/mg at 25°C, at 50% yield, compared to approximately 250 U/mg of commercial bovine heart LDH [22].

The dye-ligand bearing *p*-aminophenyloxanilic acid as biomimetic moiety and used successfully for the individual purification of each of MDH [20] and LDH [22], was also used in a facile procedure for the successive purification of mitochondrial MDH and LDH from the same bovine heart extract [23]. This procedure leads to pure enzymes, mitochondrial MDH (SA>1330 U/mg, yield≅45%) and LDH (SA≅500 U/mg, yield≅40%), as shown in Fig. 6.

The mercaptopyruvic-biomimetic ligand (BM6), when immobilized on agarose, is also effective for the purification of LDH from the fish *Pagellus erithrinus* in two chromatography steps (SA>700 U/mg, recovery>70%) [29].

The biomimetic dye-ligand bearing *p*-aminophenyloxanilic acid (BM5) was originally designed for MDH [17]. Therefore, Labrou et al. [18] designed a new biomimetic dye-ligand for LDH (Fig. 7). The situation with LDH was similar to that with MDH but not the same, as explained bellow and shown in Fig. 7.

Here again, the anthraquinone moiety of the new ligand mimics the adenine moiety of NAD^+, and it is accommodated in a hydrophobic crevice formed by five chain regions of LDH. The bottom of the cleft is blocked by Tyr-85 (not shown) and Ile-123 so the anthraquinone cannot enter deeper in the crevice. As with MDH, this results in the exposure of the sulfonate, amino and one of the carbonyl groups of the anthraquinone all pointing towards the solvent, while the carbonyl group on the other side of the anthraquinone is placed within hydrogen bonding distance of Asp-53 (Fig. 7) and Lys-58 (not shown).

The sulfonate group of the bridging diaminobenzosulfonate is located in a polar cavity where hydrogen bonds can be formed with the main chain nitrogens of Val-29 and Gly-30 (Fig. 7). The bridging amino group on the benzene ring forms a stabilizing hydrogen bond with Asp-53.

The triazine ring of the dye-ligand binds in the region where the pyrophosphate of NAD^+ binds, with the one side close to the surface of the enzyme.

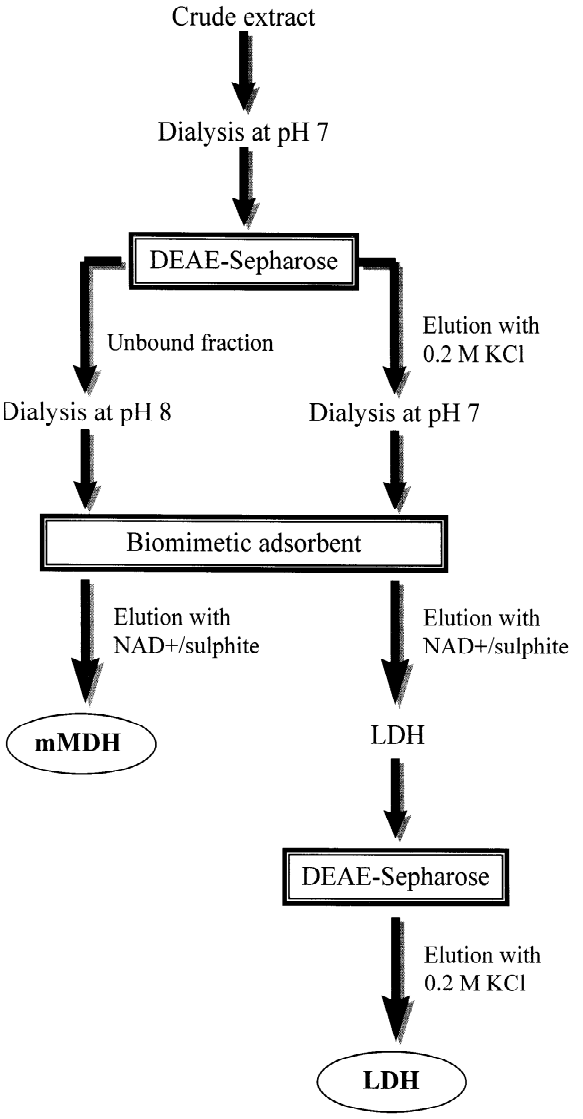


Fig. 6. The purification protocol of mitochondrial MDH and LDH from bovine heart extract.

On the other side of the triazine ring, towards the inner of the binding site, it is linked the terminal biomimetic moiety via an –NH– group, within a hydrogen bond distance from the hydroxyl group of Thr-97 (Fig. 7).

The biomimetic moiety of the dye bears a benzene ring connected via an amino group to the triazine ring. The terminal charged carboxyl group present

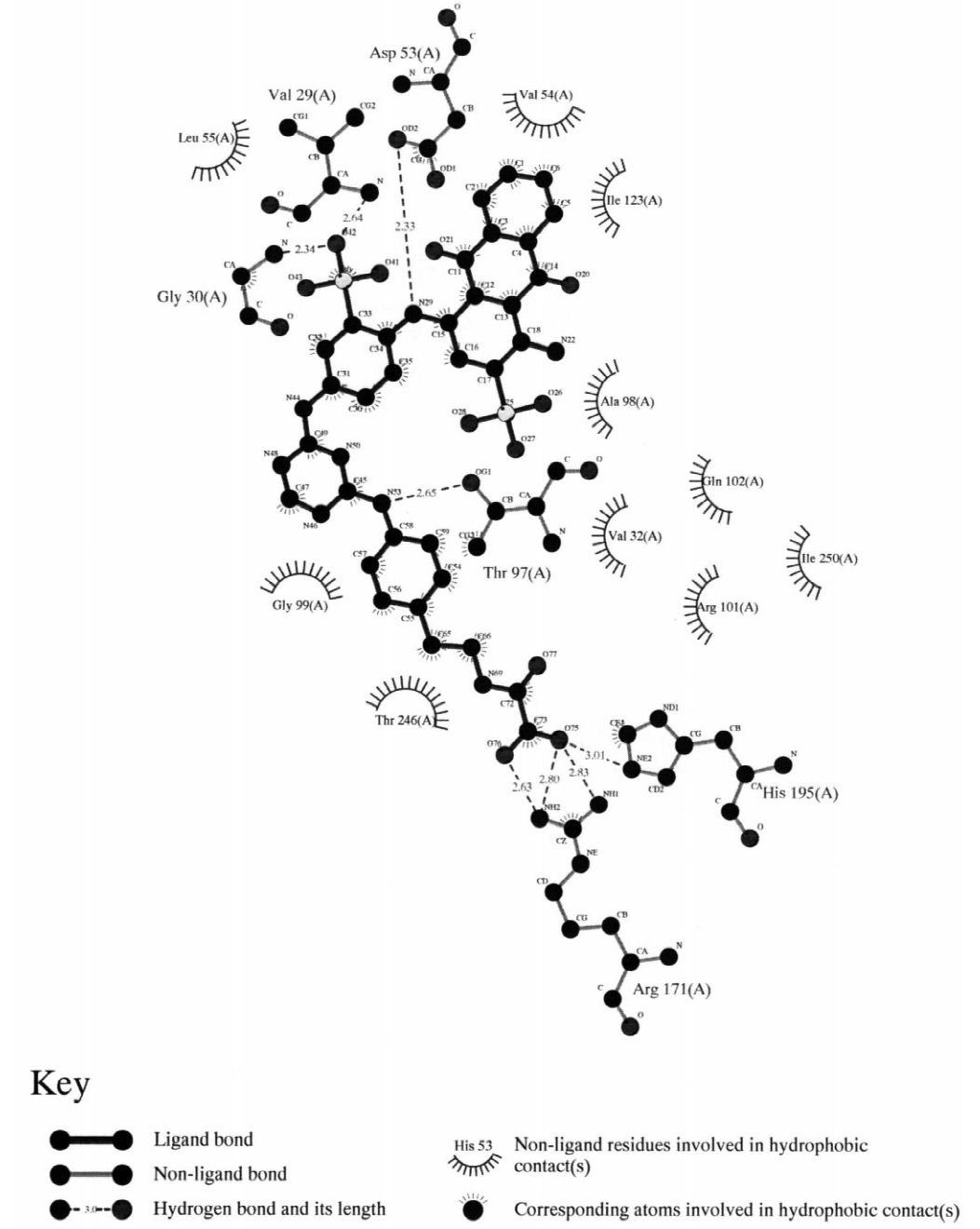


Fig. 7. Diagram of the anthraquinone ketocarboxyl-biomimetic dye in the coenzyme-binding site of pig heart LDH (courtesy of Dr. E. Eliopoulos, AUA).

was chosen to emulate the natural substrate of LDH and be able to accommodate its interactions with Arg-171. The benzene ring is placed in the space where the nicotinamide-linked ribose of NAD^+ occupied, in the hydrophobic cavity formed by Val-32, Val-138, Thr-246, Ile-250, Gln-102, the side group of Arg-101 (Fig. 7), and fragments of the main chain of LDH. The terminal carboxyl group is linked with the benzene ring via an ethylenamide spacer, $-CONH(CH)_2-$. These are positioned next to the catalytic site where the cofactor's nicotinamide is located. The carboxylate is surrounded be several polar amino acids, Gln-102, Arg-109, Arg-171, Asn-166, His-195 and Thr-246. In the case of the non-biomimetic dye CB3GA (control dye-ligand), the charged bulky sulfonate group of the terminal amino-benzosulfonate is placed against the hydrophobic Val-32 and Gln-102 in a rather restricted volume to accommodate the sulfonate group. The positioning of the terminal ketoacid of the biomimetic moiety in the catalytic site was modeled to mimic that of the natural substrate (*S*-lactate) and the terminal amide of NAD^+. This is realized by introducing aliphatic and amide groups at the *para* position of the benzene ring. From the molecular model (Fig. 7) is evident that without a spacer possessing H-bond ability after the benzene group, the ketoacid terminal group cannot reach the polar catalytic site of LDH. Therefore, the introduction of an ethyl spacer placed the ketoacid out of the hydrophobic part of the binding site, while introduction of an additional amide brought the ketoacid into the catalytic site. Therefore, employment of a benzene ring and an ethylenamide group conserves the direction of the biomimetic chain and introduces the maximum length. The latter enhanced the possibility of additional hydrogen bonds to the amine donor and carbonyl acceptor.

The practical applicability of molecular modeling and bioinformatics for dye-ligand design was assessed in the laboratory [18]. The immobilized new biomimetic dye showed a superior purifying ability for LDH from bovine heart (14.5-fold purification), compared to commercial CB3GA (7.6-fold purification) immobilized also on agarose. This superior purifying ability of the new biomimetic adsorbent for LDH was demonstrated also with five additional sources (Table 3), indicating its wide applicability.

Table 3
Summary of affinity chromatography of crude LDH from different biological sources on immobilized dyes 2-(4-amino-phenyl)ethyloxamic-VBAR and Cibacron blue 3GA[a]

Extract	SA (U/mg)		Purification (-fold)	
	BM[b]	CB3GA[c]	BM	CB3GA
Chicken liver	57.4	44.0	23.1	17.7
Chicken muscle	259.3	185.2	11.2	8.0
Pig muscle	498.3	384.5	17.1	13.2
Bovine pancreas	18.7	15.5	14.1	11.7
Pea seeds	1.21	0.65	98.0	53.2

[a] Equilibration buffer: 20 m*M* MOPS–NaOH buffer, pH 7, containing 5 m*M* β-MeSH. Desorption of bound LDH: 5 m*M* NADH. (Data were obtained from Ref. [18]).
[b] BM, 2-(4-aminophenyl)ethyloxamic-VBAR immobilized on agarose.
[c] CB3GA, Cibacron blue 3GA immobilized on agarose.

The new biomimetic adsorbent, when was integrated in a two-step purification procedure of LDH from bovine heart extract, produced pure enzyme of specific activity equal to 600 U/mg, as judged from the single band obtained after SDS–PAGE analysis (Table 4) [18].

3.4. Biomimetic ligands for galactose-recognizing enzymes

Recent examples of computer-aided ligand design are two anthraquinone galactosyl-biomimetic dyes bearing suitable galactose analogues linked via the C-1 and C-2 sugar positions [30]. These two dyes were designed for two galactose-recognizing enzymes, galactose oxidase and galactose dehydrogenase, respectively. The former enzyme acts on the C-6 position of galactose and the later on the C-1 position. The new biomimetic dyes are able to discriminate between these enzymes, since each dye recognizes and binds selectively one of the two targeted enzymes. The bound enzyme can be desorbed from the respective affinity column biospecifically in high purity and yield [30]. Molecular modeling has been employed also for the design of triazinyl non-dye ligands for protein A [31], human IgG [32] and an insulin precursor [33]. Biomimetic dyes and ligands in general, are expected to continue to play an important role as affinity chromatography tools in protein purification.

Table 4
Summary of purification procedure of LDH from bovine heart (data were obtained from Ref. [18])

Step	SA (U/mg)	Purification recovery (-fold)	Recovery (%)
Crude extract	13.5	1.0	100
DEAE-Sepharose ion-exchange chromatography (KCl elution)	65.9	4.9	70
Biomimetic-dye affinity chromatography[a] (NAD^+/sulfite elution)	600	44.4	56

[a] Immobilized 2-(4-aminophenyl)ethyloxamic-VBAR.

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