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Affinity gel interactions of alcohol and polyol dehydrogenases: anomalous behaviour and structural correlations

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SUMMARY

The initial interaction of horse and sheep liver polyol (sorbitol) dehydrogenase (L-iditol:NAD 2-oxidoreductase, EC 1.1.1.14) with the affinity gel Blue Sepharose CL-6B is anomalous and shown to be driven by an increase in entropy. In all other affinity gel interactions examined using polyol dehydrogenase with AGAMP and AGNAD and horse liver alcohol dehydrogenase (EC 1.1.1.1) with these three gels, an increase in temperature facilitated release of enzyme from the gel to the mobile phase. A more extensive evaluation of polyol dehydrogenase–Blue Sepharose CL-6B interaction points to a spectrum of forces whose individual contributions to complex formation are subject to manipulation by a change in time, temperature and/or solvent. These results can be explained by the two-stage thermodynamic model of protein–ligand association proposed by P. D. Ross and S. Subramanian [*Biochemistry*, 20 (1981) 3096]. Comparisons based on hydropathy plots with secondary and tertiary [J. Jeffery and H. Jornvall, *Adv. Enzymol.*, 61 (1988) 47] structure alignments of polyol dehydrogenase fitted to alcohol dehydrogenase were used to reveal major differences at residues 271, 47 and 202 which could account for the disparate thermal behaviour of these enzymes on Blue Sepharose CL-6B.

INTRODUCTION

Jornvall *et al.*[1] and Eklund *et al.*[2] have demonstrated that close structural similarities exist between sheep liver polyol (*i.e.*, sorbitol) dehydrogenase (EC 1.1.1.14) (SLPDH) and yeast and horse liver alcohol dehydrogenase (EC 1.1.1.1) (YADH and HLADH, respectively). This assessment is derived from secondary structure predictions[1] and projections for the tertiary structure of the polyol dehydrogenase (PDH) subunit based on model building and substrate docking using computer graphics[2].

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Parallel with these structural and mechanistic similarities there is a consensus recognition that significant compositional differences exist between SLPDH and HLADH. Maret and Auld[3], reporting on the characterization of human liver polyol dehydrogenase (ULPDH), have noted several of them: (i) SLPDH is a tetramer rather than a dimer; (ii) it contains one instead of two zinc atoms per subunit; (iii) a cysteine ligand of the catalytic zinc in HLADH is thought to be replaced by a glutamate residue.

Contrasted with these structural differences two recent reports point to the unusual behaviour displayed by PDH when this enzyme is ligated to NAD[4] and Cibacron Blue 3GA[5]. In one study Gronenborn *et al.*[4], using transferred nuclear Overhauser enhancement measurements, reported an unusual conformation of NAD bound to SLPDH *vis-a-vis* the extended conformation found in several other NAD–dehydrogenase complexes, including HLADH.

In another investigation[5], dealing with the purification of horse liver polyol dehydrogenase (HLPDH), it was observed that unlike HLADH the interaction between Blue Sepharose CL-6B (BSCL-6B) and HLPDH is unusual in that an increase of temperature enhances enzyme–gel interaction. This property was exploited in the purification of HLPDH. Harvey *et al.*[6] in a series of thermal affinity gel studies, noted that an increase in temperature decreased the affinity of yeast alcohol dehydrogenase and glycerokinase for N^6-(6-aminohexyl)-5′-AMP-Sepharose. Subsequently this same group reported[7] that an elevation in temperature increased the affinity of *Bacillus stearothermophilus* alcohol dehydrogenase and phosphofructokinase for this gel. Similarly, haptoglobin is bound to immobilized Cibacron Blue at 25°C, whereas at 4°C it is not[8]. The fact that the majority of affinity gel experiments are performed under isothermal conditions could mask the possibility that entropically driven protein–gel interactions are more common than is generally realized.

In this paper we describe the results of our studies on the similarities and differences displayed by PDH towards the affinity gels, AGAMP, AGNAD and BSCL-6B relative to HLADH, using a dual-temperature probe. The structural regions and amino acids within these proteins which could possibly account for their dissimilar thermal behaviour with BSCL-6B are examined.

EXPERIMENTAL

Materials

AGAMP Type 3 (AGAMP/3) and AGNAD Type 1 (AGNAD/1) were procured from PL Biochemicals (now Pharmacia Fine Chemicals). BSCL-6B was obtained from Pharmacia. The ligand of this gel, the textile-dye Cibacron Blue 3GA (CB-3GA), is a mixture of *m*- and *p*-isomers. It has the structure 1-amino-4[[4-[[4-chloro-6-[[3(or 4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid (CAS No. 12236-82-7). (The *o*-isomer of this dye contains a terminal 2-amino sulfonate ring and is designated CB-F3GA[9,10].) HLPDH was prepared as described[5]. All other reagents and chemicals, unless noted, were purchased from Sigma.

Methods

Chromatography. Affinity gel chromatography was carried out on column beds

(10.0 cm × 0.9 cm) of gel media previously equilibrated with 20 m*M* Tris–HCl buffer, pH 7.5, 50 m*M* in NaCl and 3.0 m*M* in dithiothreitol (DTT). Calibrated thermistors attached to the columns were used to monitor temperature. Fractions of 5.0 ml were collected (4°C) at a flow-rate of 1.0 ml/min.

Protein measurement. Protein assays were carried out using the method of Bradford[11] or Whitaker and Granum[12].

Enzyme measurements. Dehydrogenase activities were measured at 25°C in 20 m*M* Tris–HCl buffer, pH 9.2, by following the increase in absorbance at 340 nm. A typical assay system contained sorbitol (0.120 mmol) or alcohol (1.7 mmol), NAD (0.0024 mmol) and an aliquot (0.025–1.0 ml) of column effluent as the enzyme source in a total final volume of 3.00 ml. A unit of enzyme activity is reported as the number of micromoles of NADH produced per minute.

Structure comparisons. Hydropathy profiles[13] for HLADH and SLPDH were constructed using the Lotus® 1-2-3® spreadsheet program of Vickery[14].

RESULTS AND DISCUSSION

Thermal affinity gel studies

The dual-temperature release patterns of HLADH and PDH from AGNAD/1, AGAMP/3 and BSCL-6B reveal marked similarities and differences in this two-enzyme, three-gel comparison (Table I). Both enzymes show a similar temperature-insensitive interaction with AGNAD/1 at 4 and 30°C. Although the relative recoveries differ (*ca.* 40% for HLADH, *ca.* 25% for HLPDH), the bulk of the enzyme activity in both cases (*ca.* 60% for HLADH, *ca.* 75% for HLPDH) remains column-bound and is not released with a pulse of NAD.

In contrast, HLADH and HLPDH interactions with AGAMP/3 and BSCL-6B are temperature-sensitive, but with some noteworthy differences. Thus both HLPDH and HLADH interactions with AGAMP/3 are exothermic in that an increase in temperature leads to a higher relative concentration of each enzyme in the mobile

TABLE I

ENZYMATIC RECOVERY PATTERNS FOR HORSE LIVER ALCOHOL AND POLYOL DEHYDROGENASE FROM SPECIFIC AND GENERAL AFFINITY GEL LIGAND GELS AS A FUNCTION OF TEMPERATURE

(1) In the solvent front (*i.e.*, void volume) unbound by the gel after a 30-min stand; (2) subsequently released with a pulse of 10 m*M* NAD; (3) total. Tris–HCl (pH 7.5, 50 m*M* in sodium chloride, 3 m*M* in dithioerythreitol) was used as irrigant/eluent buffer. See text for commentary.

Enzyme	*Temperature (°C)*	*Percentage of enzyme activity recovered (± 7%)*								
		BSCL-6B			*AGAMP/3*			*AGNAD/1*		
		1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
HLADH	30	80	10	90	70	25	95	10	30	40
	4	5	90	95	0–5	80	85	10	30	40
HLPDH	30	5	90	95	70	20	90	0–5	20	25
	4	27	70	97	45	50	95	0–5	20	25

phase[6]. However, the interaction HLADH–AGAMP/3 is the more sensitive of the two (0–5% → 70%, *versus* 45% → 70%), suggesting this adsorption is the more exothermic of the two[6]. In all of these cases recovery of enzyme activity is essentially complete (*i.e.*, ≈85–95%).

The most striking difference in these patterns involves the reactions of HLADH and HLPDH with the BSCL-6B. Here HLADH–BSCL-6B adsorption is exothermic, resembling HLADH–AGAMP/3 interaction. In contrast, HLPDH–BSCL-6B adsorption is endothermic in that an increase in temperature enhances enzyme–gel interaction. Like AGAMP/3, but unlike AGNAD/1, full activity (≈95–97%) is recovered following the pulse of NAD.

In view of the unusual interaction of HLPDH with BSCL-6B,shown in Table I, a dual-temperature study, using SLPDH and HLADH, was carried with this gel (Fig. 1). Addition of SLPDH to BSCL-6B at 30°C shows that the bulk of this protein is bound by the gel. Only a small amount (*ca.* 10%) is recovered in the breakthrough volume. Bound enzyme is subsequently released at 4°C by salt (*ca.* 18%), followed by a pulse of NAD (72%) (Fig. 1A). When added at 4°C, the majority (*ca.* 95%) of the

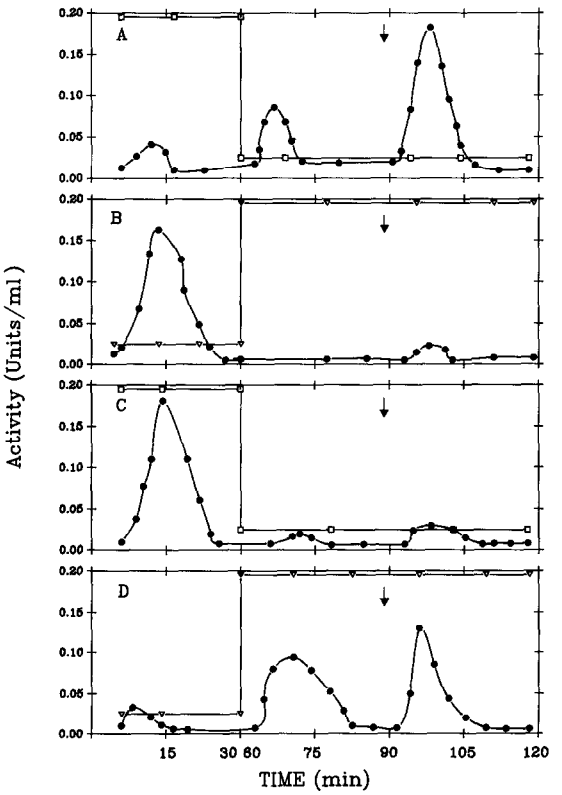

Fig. 1. Chromatography of SLPDH and HLADH on Blue Sepharose CL-6B. Samples were loaded at 30°C (□, incubator, A and C) or 4°C (▽, cold box, B and D) and eluted immediately. The columns were then reequilibrated to the alternate temperature (30 min) and the elution was resumed. The area (%) under each curve (see text) was taken as the measure of activity recovered in the individual peaks. (A and B) SLPDH; (C and D) HLADH. (●) Enzyme activity. (↓) The point of addition of a pulse of NAD.

SLPDH activity appears in the void volume. A residual amount (*ca.* 5%) is released by a pulse of NAD (10 m*M*) (Fig. 1B). Interaction of HLADH with BSCL-6B at 30°C is unlike that of SLPDH (see Fig. 1A). Here the majority of the activity (*ca.* 78%) appears in the breakthrough volume. Bound HLADH is subsequently released at 4°C by salt (*ca.* 4%) followed by a pulse of NAD (17%) (Fig. 1C). If HLADH is loaded at 4°C under the conditions described for SLPDH (see Fig. 1B), *ca.* 5% appears in the void volume. The remainder is released at 30°C by salt (*ca.* 55%) followed by a pulse of NAD (*ca.* 35%) (Fig. 1D).

The same trends described for Tris buffer were observed when the elutions were carried out in phosphate buffer (in both cases the eluting buffer contained 50 m*M* sodium chloride). For these conditions the release patterns are not significantly influenced by the larger $\Delta pK/\Delta T$ of Tris relative to phosphate (-0.028 *versus* -0.003). In all experiments described the enzymes were eluted within 1.5–2.0 h of addition to the columns. Prolonged exposure (4–6 h) led, in all cases, to enzyme entrapment and irreversible binding.

A more extensive evaluation of HLPDH–BSCL-6B interaction points to a spectrum of forces whose individual contributions to complex formation can be manipulated by a change in time, temperature and/or solvent (Table II, experiments 1–5, interacting species A–D).

Experiment 1A. At 4°C HLPDH is not bound by the gel, since *ca.* 90% of the enzyme activity is recovered in the effluent, if eluted immediately. This behaviour mirrors that of SLPDH (Fig. 1B).

Experiment 1B. A 30-min hold at 4°C enhances enzyme–gel interaction since only 31% of the activity appears in the breakthrough volume.

Experiment 1C. At 30°C, with a 30-min hold 50 m*M* sodium chloride releases

TABLE II

THERMAL RELEASE PATTERNS OF HORSE LIVER POLYOL DEHYDROGENASE FROM BLUE SEPHAROSE CL-6B

Experiment No.	*Eluting buffer*[a]			*Percentage of enzyme activity recovered (± 7%)*			
	NAD (mM)	*NaCl (M)*	*Ethylene glycol (%)*	*Protein–gel complex*[b]			
				A	*B*	*C*	*D*
1	0	0.05	0	90	31	3–5	0
2	0	0.05	50	95[c]	95	0	0
3	10	0.05	0	95	95	95	0
4	0	0.20	0	95	95	95	0
5[d]	0	1.00	0	95	95	95	95

[a] Eluting buffer is Tris–HCl (20 m*M*), pH 7.5, 3.0 m*M* in DTT with the additive(s) (NaCl, NAD or ethylene glycol) indicated.

[b] Refers to the interacting species A, B, C or D formed at 4 or 30°C between BSCL-6B and the enzyme. All samples were added to the columns at 4°C. They were then released immediately (A), held for 30 min at the stated temperature, then released [(B) 4°C; (C) 30°C] or released following treatment of C with 50% ethylene glycol at 30°C (D).

[c] Recovery of ≈90–95% or greater is taken to mean full recovery of the enzyme in the effluent.

[d] Recovery here is based on protein measurement, since HLPDH is not active in 1.0 *M* NaCl.

only a negligible amount (*ca.* 3–5%) of the enzyme. The same pattern is seen for SLPDH (Fig. 1A).

Experiment 2B. When ethylene glycol (50%) is incorporated in the eluting solvent at 4°C recovery is boosted to 95%.

Experiment 2C. Exposure of the complex at 30°C (following a 30-min hold) to 50% ethylene glycol profoundly enhances enzyme–gel binding such that the "all" release pattern (95%, Experiment 2B) is changed to "none".

Experiments 1D–5D. This HLPDH–BSCL–6B species was refractory to all subsequent release attempts, except with 1.0 *M* sodium chloride.

The void volume recovery noted for HLPDH at 4°C (27%, Table I) from BSCL-6B compares favorably with the value (31%) cited in Experiment 1B (Table II). Similarly, the recovery reported for SLPDH at 4°C (95%, Fig. 1B) is comparable to that cited for HLPDH (90%, Experiment 1A, Table II). These results demonstrate the profound influence of the contact time variable on the gel–enzyme release patterns at 4 or 30°C.

Release patterns displayed here (Table II) are consistent with the interpretation that ethylene glycol diminishes hydrophobic interaction and strengthens electrostatic interaction; that salt weakens electrostatic interaction and strengthens hydrophobic interaction[15]. Elevated temperature and prolonged exposure time enhance hydrophobic interaction.

The gel–enzyme interactions described in Table II are in accord with the two-step thermodynamic model proposed by Ross and Subramanian[16] to describe protein–ligand associations. Thus A corresponds to the individually hydrated species (in this case HLPDH and BSCL-6B) which in B or C partially interact so that there is a mutual penetration of their hydration layers to form a "hydrophobically associated species". For this transformation (A → B or C) both ΔH^0 and ΔS^0 are positive and ΔG^0 is negative. In step 2 (B or C → D) the presence of ethylene glycol at 30°C weakens the hydrophobic association and, at the same, time enhances "intermolecular interactions" which include ionic, hydrogen bond and Van der Waals forces. The solvent molecules are not thought to play a significant role in this step.

Structure comparison studies

In what follows we compare the results of our thermal affinity gel data and (i) the hydropathic characteristics of these enzymes with, (ii) their tertiary structure alignments[2,17] and (iii) the results derived from X-ray studies dealing with HLADH and CB3GA interaction sites[18]. This assessment points to the fact that only a very few (one to three) key amino acid differences between HLADH and PDH may be sufficient to account for the dissimilar dual-temperature gel interactions reported here.

From hydropathy plots. When the amino acid composition of HLADH[19] is ranked with SLPDH[20] (and ULPDH[3]) on the basis of residue apolarity[21], no differences between these proteins are evident (data not shown). However, some insight to the location of HLADH and SLPDH site differences can be gained from a comparison of Kyte and Doolittle[13] hydropathy plots of these enzymes (Fig. 2).

Thus the amino acid segment *ca.* 45–70 in SLPDH is a hydrophilic region leading up to a weak hydrophobic area. The comparable segment in HLADH begins with a *ca.* 10 amino acid stretch not showing an apolar or polar bias and closes with the build-up of a strong hydrophobic region. Within the sequence *ca.* 145–180 SLPDH

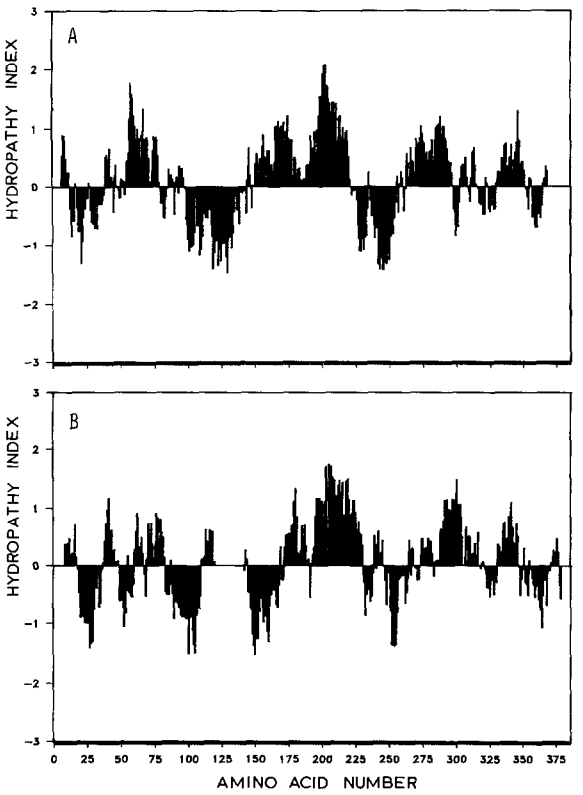

Fig. 2. (A) Hydropathy plot of HLADH showing a 13 amino acid average segment plotted against the amino acid number. Regions above the (universal) ≈zero midline are designated hydrophobic (*i.e.*, interior) spans, those below identify hydrophilic (exterior) spans. The boundary of the catalytic domain is defined by amino acids ≈1–175 and ≈319–374. The coenzyme binding domain is localized over amino acids ≈176–318. (B) Hydropathy plot of SLPDH showing a 13 amino acid average segment plotted against the HLADH amino acid number. The gaps that appear in the SLPDH amino acid profile arise from an aligment utilizing the computer-based model generated from the conserved tertiary structure of HLADH[2]. Regions above the (universal) ≈zero midline are designated hydrophobic (interior), those below hydrophilic (exterior).

is judged to be predominantly hydrophilic, while HLADH is characterized as hydrophobic. A third regional difference is centered on segment *ca.* 260–285. Here HLADH is strongly hydrophobic while the corresponding SLPDH segment is classified as indifferent to weakly hydrophobic.

From tertiary structure alignment. When the individual amino acids in the retained tertiary structure of HLADH are replaced with the corresponding SLPDH amino acids relatively few residues are altered in the NAD coenzyme binding domain[2,22]. These results are summarized in Table III. Major differences are seen to occur within three of the regions identified in the hydropathy plots (at positions 271, 47 and 174) and at residue 202. Within this preserved three-dimensional alignment, amino acids 271 and 202 are found to lie in relatively close proximity to each other

TABLE III

NAD AND CB3GA INTERACTION SITES WITH HLADH AND SLPDH

Amino acid				*Interaction sites*	
Residue No.[a]	*ADH*[b]	*PDH*[b]	*Diff.*[c]	*NAD*[d]	*CB3GA*[e]
224	I	L	m	N1A, C2A, N3A, C4A, C5A, C6A	(D) Anthraquinone ring
271	R	V	M	N6A	(C) Bridging 3-sulfophenyl ring
269	I	T	m	N3A, C4A, C5A, N7A, C8A, O4'A, C3'N, O3'N, C4'N	
223	D	D	o	C2A, N3A C1'A, C2'A, O2'A, O3'A, C3'A, O4'A	
199	G	G	o	N3A, O4'A	
228	K	R	m	O2'A, C3'A, O3'A	
201	G	G	o	O3'A, OP2A, OP2N	
47	R	G	M	OP1A OP3 OP1N, C3'N	(B) Triazinyl ring
202	G	P	M	OP2A, OP2N	
369	R	K	m	OP2N, OP1N	(A) Terminal 4-sulfophenylamino ring
203	V	I	m	OP2N, C5'N O5'N, C5N, C6N	
51	H	H	o	O2'N, O3'N	
48	S	S	o	O2'N	
178	T	V	m	C4N, C5N	
46	C	C	o	OP1N, C5N, C6N	
174	C	E	M	C4N	

[a] Numbers refer to the residues of HLADH or SLPDH that interact with NAD. Alignment is based on computer model fitting to the tertiary structure of HLADH[2].

[b] Single-letter amino acid identities (see Table III) of HLADH and SLPDH with site number referenced to HLADH[2].

[c] Designates residue differences: M = major; m = minor; o = none.

[d] Descriptors of NAD atoms closer than 0.38 nm to HLADH[22] for amino acid residues identified in the first column. See also Fig. 3.

[e] D→A Refer to the Cibacron Blue ring identities cited by Biellmann *et al.*[18] (see Fig. 3).

towards the purine ribose side of the cofactor binding domain (see Fig. 2 of ref. 23 or Fig. 6a of ref. 22).

Though few, these residue substitutions could lead to profound changes in the character of ligand binding. Thus the alteration Arg271 → Val271 leads not only to a loss of positive charge at the rim of the adenine binding pocket[2], but also to a loss of the putative binding site for the bridging 3-sulfophenyl (C) ring of CB3GA[18] (see Fig. 3 and the section *From X-ray studies*).

The transformation Gly202 → Pro202 precludes hydrogen bond formation of the main-chain nitrogen residue 202 with cofactor phosphate oxygen. Similarly, the replacement Arg47 → Gly47 results in the loss in SLPDH of one charged hydrogen

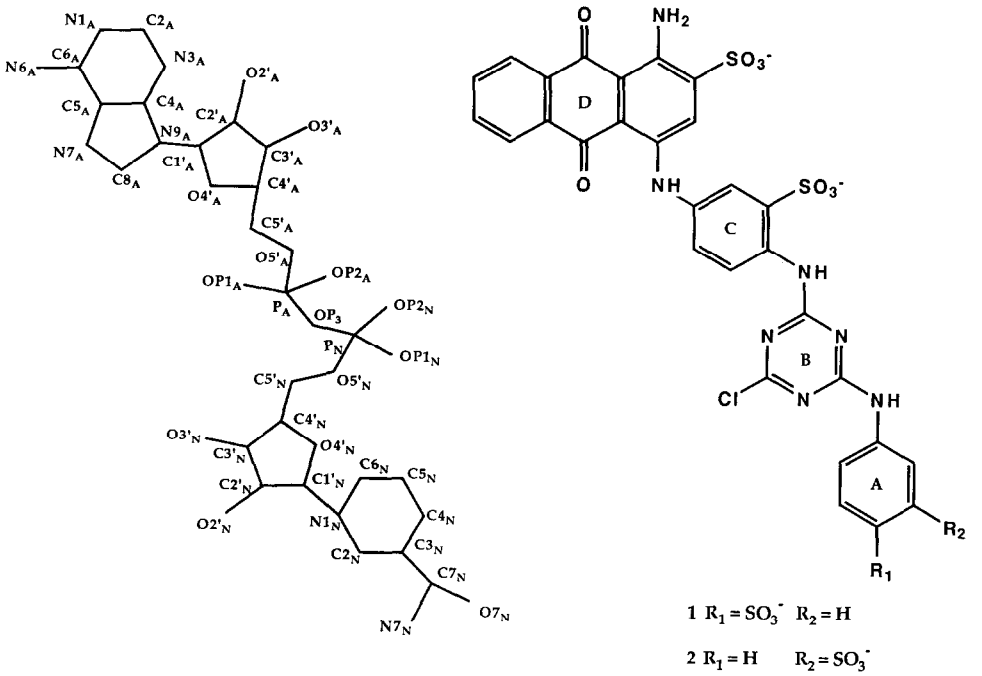

Fig. 3. (Left) Structural display of NAD^+ including the atom descriptors cited in Table III (column 5). Adapted with permission from ref. 22. © (1984), American Chemical Society. (Right) Chemical structure of Cibacron Blue 3GA with the ring identities cited in Table III (column 6). Reproduced with permission from ref. 18. © (1979), Springer-Verlag.

bond interaction between the (Arg) side-chain and one phosphate of NAD. Finally, the change Cys174 → Glu174 arises by default as a consequence of the preserved HLADH conformation at the nicotinamide catalytic site[2]. It is not thought to be involved in the binding described here, as judged by the dye–HLADH X-ray studies[18].

From X-ray studies. These experiments[18] (summarized in Table III and Fig. 3) reveal that the anthraquinone (D) ring of the dye (*p*-isomer) binds in the wide apolar pocket (of HLADH) which at one end binds the adenine segment of the NAD coenzyme. The position of the bridging 3-sulfophenyl (C) ring is such that it could interact with the ADH guanidinum group of Arg271. The triazinyl (B) ring binds in the region where the NAD pyrophosphate moiety binds with the chlorine atom close to the nicotinamide ribose binding site. From then on CB3GA binds quite differently from NAD. The terminal 4-sulfophenylamino (A) ring is bound in the left between the domains with a possible interaction of the sulfonate group with Arg369. The midpoint of this ring differs from the midpoint of bound nicotinamide by about 1 nm. Thus the binding of Cibacron Blue to HLADH partially resembles NAD binding in the ADP part, but differs greatly in the remaining parts[18]. The affinity gel binding patterns of HLADH and PDH described here (Table I and Fig. 1) are in accord with the results from these X-ray studies.

CONCLUSIONS

Among the AGNAD, AGAMP and BSCL-6B affinity gel alcohol or polyol dehydrogenase interactions reported here, the AGAMP and BSCL-6B binding patterns with these enzymes reveal a thermal dependence not evident in the AGNAD–enzyme interactions. This behaviour is indicative of hydrophobic association, since among the various intramolecular interactions, only hydrophobic interactions are strongly temperature-dependent[24]. Further, all interactions are shown to be exothermic, except the BSCL-6B–PDH association, which is driven by an increase in entropy. This binding pattern is similar to the HLADH–CB3GA association revealed by X-ray studies[18] where the anthraquinone ring binds in the hydrophobic pocket reserved for the purine-ribose segment of NAD. The substitutions Lys228 → Arg228 and Arg369 → Lys369, although classed as minor (m, Table III), will undoubtedly contribute to altered hydrogen bond and bound-water patterns in their local environments[25]. However, structure comparisons between SLPDH and HLADH based on alignment studies suggest that the focus of attention for future experiments could best be directed to residues 271, 47 and 202 (based on HLADH amino acid numbers) as the most obvious targets for PDH site-specific mutation experiments designed to account for the differences reported here.

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