tronic ground state was observed; this is especially interesting since the Mg atom has the closed-shell configuration (−−−$3s^2$).

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Chemistry and Structure of Alcohol Dehydrogenase: Some General Considerations on Binding Mode Variability

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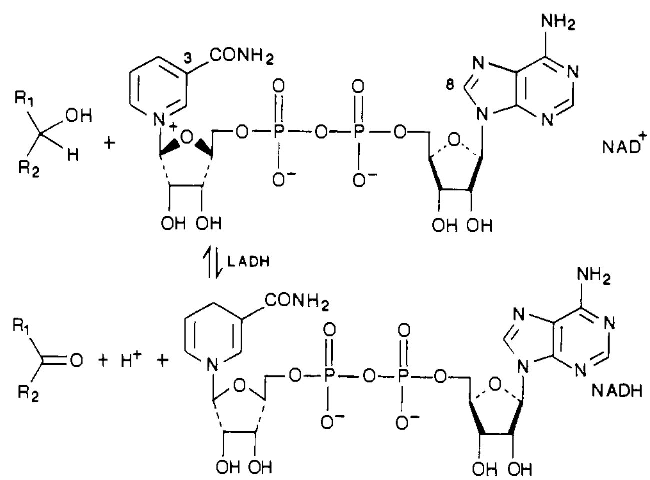
Liver alcohol dehydrogenase (LADH) (E.C.1.1.1.1) provides a superb example of collaborative interaction between protein crystallographers and bioorganic chemists.[1] During the stages of the structural determination of its isoenzyme EE, the collaboration evolved from preparing compounds for use as tools in the structure determination to a stage where the enzyme structure itself was used to design molecules which might bind to the enzyme. In this Account we describe work involving the crystallography group of Dr. C. I. Bränden (Uppsala) and our own group in Strasbourg.[2]

Liver alcohol dehydrogenase belongs to the major pathway of alcohol metabolism[3] (Scheme I). No other physiological role has been firmly established for this enzyme. The enzyme is a dimer composed of two identical subunits, each containing two zinc ions. One of these ions plays a structural role. The other, called "active site zinc", plays a role in the oxidation–reduction as a Lewis acid. It is coordinated to Cys-46 and -174 and His-67. Alcohol dehydrogenase belongs to the group of metal-containing NAD(P)$^+$-dependent dehydrogenases, which also includes sorbitol dehydrogenase[4] and glycerol dehydrogenase.[5]

The structure of LADH is presented in Figure 1.[6] The two subunits interact through their nucleotide binding domains. The nucleotide binding domain presents general features which have been conserved in the evolution of the nucleotide-dependent enzymes.[7] The coenzyme NAD(H) binds to the nucleotide binding domain and the nicotinamide ring extends towards the catalytic domain. This catalytic domain determines not only the catalytic events but also the substrate specificity. The presence of a large hydrophobic barrel explains why the enzyme acts on a great variety of aliphatic and cyclic ketones, aldehydes, and alcohols,[8] represented in a diamond lattice.[9,10] The fact that the

Scheme I



J. F. Biellmann received a Doctorat-es-Sciences from the University of Strasbourg in 1961 under the direction of G. Ourisson. After postdoctoral work with W. S. Johnson and D. Arigoni, he began his independent research in Strasbourg. His research interests range from synthetic chemistry to the chemistry of enzyme structure.

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(2) Additional collaborative efforts on other aspects of LADH have involved the groups of Prof. M. Zeppezauer (Sarrebruck) and Prof. B. V. Plapp (Iowa), but the present report will be focused on areas of interest to the Strasbourg group.

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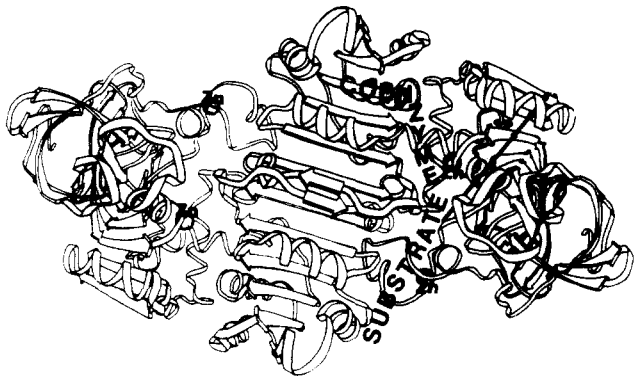
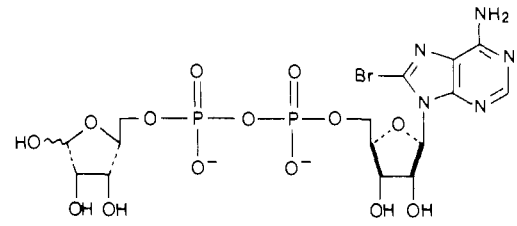


Figure 1. Schematic drawing of the LADH dimer, designed by Bo Furugren. The central part of the molecule illustrates the two coenzyme binding domains bound together across a 2-fold axis perpendicular to the plane of the paper. The two catalytic domains are separated from this central part by the coenzyme and substrate binding clefts. The binding sites for the coenzyme and substrate have been indicated for one of the subunits. The metal binding sites are located at the fringes of the catalytic domains. The transition of LADH from the apo to the holo conformation takes place through a domain rotation (6).

coenzyme is not firmly bound to the enzyme makes it easy to study the interactions of coenzyme analogues.[11,12]

Determination of the Coenzyme Binding Site

8-Bromoadenine Derivatives. The apoenzyme of LADH crystallizes in the orthorhombic system; the addition of the coenzyme destroys the apoenzyme crystals.[1] Indeed the complexes of the enzyme with the coenzyme crystallize in either the mono- or triclinic system. Adenosine diphosphoribose (ADPR), which is a good inhibitor of this enzyme,[13] does not destroy the orthorhombic crystals. Since the structure determination of the apoenzyme was undertaken first, ADPR was used to detect the coenzyme binding site. But the resolution attained does not permit a completely unambiguous determination of the orientation of ADPR bound to the enzyme. Thus to ascertain the orientation of ADPR, a bromine-labeled analogue of ADPR, 8-bromoadenosine diphosphoribose (8-Br-ADPR) 1 was prepared. In analogy to ADPR this derivative inhibits LADH. From the electron density difference maps of 8-Br-ADPR-LADH and ADPR-LADH the bromine atom was located, and it was confirmed that the active site region contains one of the zinc ions.[14] Unexpectedly the conformation of the glycosidic bond of 8-Br-



8-Br-ADPR

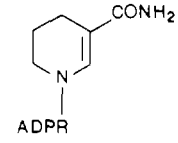
1

adenine-ribose is anti in contrast to the syn conformation observed in crystalline 8-bromoadenosine.[15] Subsequent studies on the 8-substituted analogues of adenine and guanosine showed that they are predominantly in the syn conformation in solution, but that the anti conformation is also present to a small extent.[16] This may explain why 8-Br-ADPR 1 ($K_i = 2.5 \times 10^{-4}$ M) is a weaker inhibitor than ADPR ($K_i = 2.5 \times 10^{-5}$ M) since the anti conformation of 8-Br-ADPR is less populated than for ADPR.[14]

N-8-BrAD$^+$ and *N*-8-BrADP$^+$ have been used in the structure determination of the holoenzymes of glyceraldehyde-3-phosphate dehydrogenase[17] and 6-phosphogluconate dehydrogenase.[18]

Tetrahydronicotinamide-Adenine Dinucleotide

Schellenberg's proposal and the experimental indications that the reversible hydride transfer from the substrate to the coenzyme catalyzed by NAD(P)$^+$ dependent dehydrogenases may involve the direct participation of an enzyme group[19] led us to propose that the coenzyme acts not only as redox reagent but also as an effector of conformational change necessary for redox reaction.[20a] On the basis of the geometrical similarity of the 1,4-dihydronicotinamide and 1,4,5,6-tetrahydronicotinamide rings,[21] we proposed that NADH and 1,4,5,6-tetrahydronicotinamide-adenine dinucleotide (H_2NADH) 2 may induce the same con-



H_2NADH

2

formational change in the enzyme. As expected H_2NADH 2 does not act as a coenzyme for LADH but is an inhibitor. In the presence of H_2NADH 2, no acetaldehyde-ethanol exchange was detected.[20] Schellenberg's proposal was not confirmed.

(10) However, the implication of this model may be restricted to ketones. Thus, aliphatic methyl ketones like 2-butanone and aromatic aldehydes like benzaldehyde are substrates for the enzyme. But acetophenone is, at best, a very poor substrate and does not act as an inhibitor (unpublished observation from our laboratory). The simplest explanation is that with aldehydes, the carbonyl group has an orientation different from that of the same group of structurally similar ketones such that the phenyl group of benzaldehyde is accomodated in the structure in a proper way for hydride transfer, while acetophenone does not bind at all. This could also explain the stereospecific reduction of acetaldehyde by LADH.

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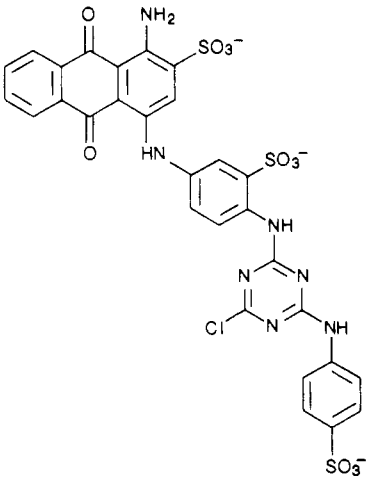
H_2NADH and H_2NADPH were very useful in demonstrating that the reduced coenzyme beside being a hydride donating group induced a conformational change to the active forms.[20b,22] In the presence of H_2NADPH, the 6-phosphogluconate and isocitrate dehydrogenase catalyse the hydrogen exchange of ribulose 5-phosphate and α-ketoglutarate. H_2NADPH also acts as an activator in the oxidation of isocitrate by isocitrate dehydrogenase.[23,24]

With LADH and *p*-(dimethylamino)cinnamaldehyde, H_2NADH forms a stable ternary complex whose spectroscopic properties are similar to those transiently observed with NADH as coenzyme.[25,26] The complex with H_2NADH **2** does not change with time as does that with NADH. The crystal structure of the ternary complex LADH–H_2NADH–*p*-(dimethylamino)cinnamaldehyde has been determined.[27] The aldehyde oxygen is coordinated to the zinc ion and the distance between the hydrogen atom at C-4 of H_2NADH and the aldehyde carbon is about 3.6 Å. Thus a complex LADH–H_2NADH–aldehyde whose structure is likely to be similar to that of the active complex LADH–NADH–aldehyde has been observed. This is possible because H_2NADH does not act as reducing species. In other instances, substrate analogues, which cannot undergo the reaction for thermodynamic reasons, may form stable complexes whose structures are close to those of the reactive complexes.[28]

The use of such inactive analogues may provide more information about the active site than can be deduced from the use of common inhibitors, since it permits the study of stabilized intermediates along the reaction pathway. For instance, can the *Z*-isomer of 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldoxime be regarded as an alcohol analogue since it forms a complex with LADH–NAD^+?[29,30]

Binding of Cibacron Blue F3GA to Enzyme

The use of Cibacron blue F3GA as a specific ligand for some enzymes raised the question of how this dye binds to proteins. The proposal that Cibacron blue F3GA binds specifically to the dinucleotide binding fold,[31] led us to examine how Cibacron blue F3GA binds to the orthorhombic crystals of LADH. The electron density map at a resolution of 0.37 nm showed that the

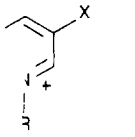


Cibacron blue F3GA

dye occupies only partially the dinucleotide binding site. The anthraquinone is bound to the rather unspecific adenine binding site. The *p*-aminophenyl sulfonate group probably interacts with the side chain of Arg-369 which is not involved in the binding of the coenzyme.[32] The good balance of lipophilic and hydrophilic groups of Cibacron blue F3GA is responsable for the good binding of this dye to LADH. We proposed that other molecules with the proper combination of lipophilic and hydrophilic groups may prove to be strong specific ligands for proteins. This has proven to be the case recently,[33] and Cibacron blue F3GA should not be regarded as nucleotide fold specific.

Pyridine and 3-Halogeno Pyridine Adenine Dinucleotide

Further information about the coenzyme binding was obtained by studying 3-iodo-, 3-bromo-, and 3-chloropyridine–adenine dinucleotide (I^3–, Br^3–, Cl^3PdAD^+) **6**, **5**, **4**, and pyridine adenine dinucleotide ($PdAD^+$) **3**. The



X=H **3**, $PdAD^+$
X=Cl **4**, Cl^3PdAD^+
X=Br **5**, Br^3PdAD^+
X=I, **6**, I^3PdAD^+

three halogenated coenzyme analogues show coenzyme activity with LADH.[29,34-36]

The binding to LADH in the crystalline state of I^3PdAD^+ **6** and $PdAD^+$ **3** was determined under such conditions that crystals of the complexes are isomorphous with apoenzyme crystals. The results were quite unexpected. Both analogues are bound in the same manner. The adenosine part of I^3PdAD^+ and $PdAD^+$ is bound in a manner similar to that of ADPR and NADH. But the remaining part of the dinucleotides

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(30) The 3-carboxyamide-phenyladenosine dinucleotide and its phosphate should be even better analogues of NAD(P)H since the puckering of the 1,4,5,6-tetrahydronicotinamide ring is absent. Phenyladenine dinucleotide has been prepared (Danenberg, P. V.; Danenberg, K. D.; Cleland, W. W. *J. Biol. Chem.* **1978**, *253*, 5886–5887). Unfortunately, there is no inactive analogue bearing a positive charge as in $NAD(P)^+$ and binding to the active site available.
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is bound quite differently at the surface of the crevice of the two domains of the subunit. The C-1 atom of the pyridine ribose of I^3PdAD^+ **6** is located 1.6 nm from the position of the same atom of ADPR. The pyridinium ring is close to Lys-228 and this region is not the active site region.[37] Modification of Lys-228 enhances the activity of LADH by increasing the coenzyme dissociation rate.[38,39] So we have detected that this analogue binds in two fashions to LADH: one outside the active site detected by X-ray structure determination and one in the active site responsible for the activity as cofactor.

5-Methylnicotinamide–adenine dinucleotide (me^5NAD^+), which does not show any activity as coenzyme with the major isoenzyme, is bound outside the active site as $PdAD^+$ and I^3PdAD^+.[40] The reason for the stabilization of the nonactive binding mode which could very well be an intermediate in the binding of the coenzyme to LADH remains unknown. The binding of these coenzyme analogues outside the active site, in addition to their active site binding required for activity, is an example of variable binding mode which is described below.

Variable Binding Mode

The often used term "wrong way binding" is probably inappropriate, because the "wrong way binding" is unpredicted binding rather than "wrong".[41] Certainly the concept of a variable binding mode is more appropriate and will be used here.

A variable mode of binding of a molecule to a macromolecule may occur in two ways: the first where two modes of binding of one molecule to a macromolecule occur in an exclusive fashion and [For instance I^3PdAD^+ binds in one manner where the 3-iodopyridinium ring is located outside the active site and in another manner where the 3-iodopyridinium ring is located in the active site.] the second where one member of a family of compounds binds differently from the rest. [For instance (5-methyl)NAD^+ binds with the pyridinium ring outside the active site and shows no activity as coenzyme in agreement with the modeling of this analogue in the LADH structure. This type of variable binding may be detected in structure activity relationships which may break down for certain compounds. An example is dihydrofolate reductase of *Lactobacillus casei* where folic acid and methotrexate do not bind in the same manner.[42,43]]

Variable binding mode has been proposed under the name nonproductive binding to explain the decrease of the catalytic rate with poor substrates.[44-47] The binding of poor substrates to the enzyme occurs in several modes among which the active complex is disfavored. A dynamic view is the induced fit where the substrate binding induces a conformational change responsable for the catalysis.[48]

Detection of the variable binding mode of small molecules to a macromolecule is not easy.[49-53] One selected case beside those discussed above will be briefly presented.

The nuclear Overhauser effect has been used to study the binding of $NADP^+$ and thionicotinamide–adenine dinucleotide phosphate (thio $NADP^+$) to *Lactobacillus casei* dihydrofolate reductase. Both are bound with a very similar conformation to the enzyme except at the pyridinium–ribose bond. The $NADP^+$ binds with the anti conformation at the glycosidic bond while for thio$NADP^+$ the distribution of syn and anti conformers is similar to that observed in solution for related compounds. The authors proposed that the nicotinamide ring extends into solution.[49] ThioNADPH is a coenzyme for the enzyme.[50] The situation is quite reminiscent of that encountered with LADH and I^3PdAD^+, $PdAD^+$ and me^5NAD^+ discussed above.

Variable Binding Mode and Affinity Labeling

Affinity labeling is a useful tool for determining the residues implicated in the active site of an enzyme.[54-57] The irreversible incorporation of the label obeys the scheme:

$$E + I \overset{K}{\rightleftharpoons} E\cdots I \xrightarrow{k} EI \qquad (1)$$

In general the equilibrium is assumed for the first step.[58,59] Pseudo-affinity labeling, in which a second order inactivation occurs in addition to the formation of a binary complex between the reagent and the protein, exhibits the same time and reagent concentration dependance as affinity labeling.[60] Pseudo-affinity labeling has been considered and excluded for the labeling of 6-phosphogluconate dehydrogenase with coenzyme analogues.[61]

$$E\cdots I \rightleftharpoons E + I \rightarrow EI \qquad (2)$$

The case of variable binding mode where several complexes between the protein and the inhibitor are formed and where these complexes may give rise to irreversible incorporation is presented in the general form (eq 3) with the incorporation rate law K_i being the dissociation constant of $E\cdots I_i$ (eq 4). Two simple and

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(60) Reference 54, pp 123–124.
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$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} E\cdots I_1 \xrightarrow{k_1} EI_1$$

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E\cdots I_i \xrightarrow{k_i} EI_i \qquad (3)$$

$$E + I \underset{}{\overset{K_n}{\rightleftharpoons}} E\cdots I_n \xrightarrow{k_n} EI_n$$

$$\text{Log}\ \frac{E_{\text{Total}}}{E_{\text{Total}} - \sum_{i=n}^{i=1} EI_i} = \frac{\sum_{i=n}^{i=1} k_i/K_i}{\sum_{i=n}^{i=1} 1/K_i + 1/[I]}t \qquad (4)$$

illustrative possibilities will be discussed. For the first, two complexes are formed and give rise to the irreversible modification:

$$\text{Log}\ \frac{E_{\text{Total}}}{E_{\text{Total}} - EI_1 - EI_2} = \frac{(k_1K_2 + k_2K_1)/(K_1 + K_2)}{1 + ((K_1K_2)/(K_1 + K_2))\ (1/[I])}t \qquad (5)$$

The rate law is very similar to that of affinity labeling, except that the constants do not correspond to a single step. If the residues labeled in EI_1 and EI_2 can be determined, the ratio of the two is

$$\frac{[EI_1]}{[EI_2]} = \frac{k_1}{k_2}\frac{K_2}{K_1} \qquad (6)$$

and does not depend on reaction time and inactivator or protein concentration.

The second possibility is that beside complexes leading to inactivation, some do not give rise to irreversible incorporation. For instance two complexes are formed and only one gives rise to irreversible incorporation.

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} E\cdots I_1 \xrightarrow{k_1} EI_1 \qquad (7)$$

$$E + I \underset{}{\overset{K_2}{\rightleftharpoons}} E\cdots I_2$$

$$\text{Log}\ \frac{E_{\text{Total}}}{E_{\text{Total}} - EI_1} = \frac{(k_1K_2)/(K_1 + K_2)}{1 + ((K_1K_2)/(K_1 + K_2))(1/[I])}t \qquad (8)$$

If the constants are determined as if the irreversible incorporation corresponded to an affinity labeling, k_{app} and K_{app} would be

$$k_{app} = \frac{k_1K_2}{K_1 + K_2} \qquad (9)$$

$$K_{app} = \frac{K_1K_2}{K_1 + K_2} \qquad (10)$$

$$\frac{k_{app}}{K_{app}} = \frac{k_1}{K_1} \qquad (11)$$

Only k_{app}/K_{app} gives information on the kinetic constants of the labeling reaction. If the second complex $E\cdots I_2$ is formed preferentially, that is $K_1 \gg K_2$, relations 9 and 10 become eq 12 and eq 13. So for the dissociation the labeling kinetics give K_2 and not K_1 the dissociation constant of the labeling reaction. Very often a similar value found for the dissociation constant determined by other means, with the label or with an isosteric compound, is taken as a strong indication in favor of an affinity labeling in the expected site, which may not be the case as shown above. The inactivation rate constant found is then k_1K_2/K_1 and is smaller than the real one, thus making it likely that the reactivity of the complex tends to be underestimated.

$$k_{app} = k_1\frac{K_2}{K_1} \qquad (12)$$

$$K_{app} = K_2 \qquad (13)$$

If related affinity labels for the same protein are available, the label which binds most strongly is often the less reactive. At first, this looks surprising, since if the detected complex is along the pathway for labeling, one might infer that the stronger complex involves closer reactive groups and, then, higher reactivity. However, if the labeling does not occur from the most favored complex, the lower reactivity of the more strongly bound label is readily understood.

For instance, the inactivation of estradiol 17β-dehydrogenase from human placenta, with 3-chloroacetylpyridine–adenine dinucleotide ($Clac^3PdAD^+$) and its phosphate ($Clac^3PdADP^+$) corresponds to an affinity labeling. The rate constants k for the reaction in the binary complex are 1.3×10^{-3} s^{-1} for $Clac^3PdAD^+$ and 3.34×10^{-4} s^{-1} for $Clac^3PdADP^+$ the more strongly bound complex. And $Clac^3PdAD^+$ gives rise to three labeled peptides and $Clac^3PdADP^+$ only one. For $Clac^3PdADP^+$, the more strongly bound affinity label, fewer complexes are formed than with the more loosely bound inhibitor, where several complexes leading to inactivation are formed.[62,63] Another example is the labeling of pancreatic elastase with peptidechloromethylketones as affinity labels.[64-66] The trifluoroacetylated inactivators bound more strongly than the acetylated ones but were less reactive. From equation 11, the constancy of the ratio k_{app}/K_{app} could mean that for the acetylated and trifluoroacetylated inactivators, the k_1 and K_1 of the inactivation were related.

Substrate-like reagents such as 1-chloro-3-tosylamido-7-amino-2-heptanone inactivate trypsine by alkylation of His-46,[67-69] and phenacyl bromide derived reagents react with Ser-183.[70] Both His-46 and Ser-183 are present in the active site.[71] Both types of reagents act as affinity labels. Since the labeling occurs at different residues in the active site and since the haloketones are similar reactive functional groups, one must assume that different binding of the affinity labels in the active site occurs.

With respect to variable binding mode, the most illustrative example is the action of an NAD^+ analogue

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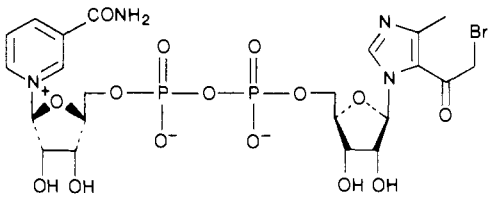
on LADH: nicotinamide–5-bromoacetyl-4-methylimidazole dinucleotide **7**. This analogue **7** is active as



7

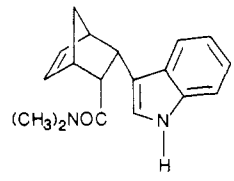
a coenzyme. Analogue **7** also acts as an affinity label[72] and labels the Cys-174 ligand of the active site zinc ion.[73] This could be an example of a variable mode of binding. The first mode of binding is related to that of NAD^+ in which the pyridinium ring is located in the active site. In the second, the molecule is bound in the inverted way. The pyridinium ring is probably in the adenine binding site and the 5-bromoacetyl-4-methylimidazole in the active site, close to Cys-174. The adenine binding site is not very specific and adenine may be replaced with a large variety of groups.[1] For instance bis(nicotinamide) dinucleotide is an active analogue of NAD^+ with LADH.[74] The symmetrical structure of NAD^+ with the two 5-phosphoribosyl units is certainly a favorable factor for the existence of the two binding modes.

Thus *the design of an affinity label is a compromise of two requirements. The first is that the modified group is really in the expected region. The second is that the affinity label, whose synthesis may be time consuming, reacts with the enzyme.* Certainly with a certain degree of freedom of the reactive group, the proper orientation favorable to a reaction may be attained, but the modified group may be outside the region of interest.

The variable binding mode has also been detected for suicide inhibition of glutamate decarboxylase by L-serine *O*-sulfate.[75,76]

Fluorescence of LADH

The fluorescence of LADH has been shown to be due to the emission from two independent classes of tryptophan.[77] The X-ray structure determination located them spatially. Trp-15 is close to the surface and Trp-314 is buried inside the protein close to the interaction domain of the two subunits through the nucleotide binding fold. The fluorescence is quenched upon binding of coenzyme.[78,79] In order to determine the contribution of the two chromophores to the fluorescence, we studied the effect on the fluorescence of quenching by iodide and by coenzyme.[80] Trp-15 fluorescence is quenched by iodide and that of Trp-317 by the coenzyme binding. The accessibility of the tryptophan residue Trp-15 through the solution explains the iodide quenching. But the florescence quenching of Trp-317 upon coenzyme binding was unexpected, since the structure determination had shown that this part of the protein is rather rigid and does not undergo a detectable conformational change on binding of any ligand to the enzyme. Radiationless energy transfer processes contributed to a small extent to the quenching, and we proposed that a small conformational change not detected in the structure determination modifies the environment of Trp-317 and thus causes fluorescence quenching. Later work on fluorescence quenching by acrylamide confirmed our results.[81] In the studies of the fluorescence decay, two exponential decays are detected, one attributed to Trp-15 and another to Trp-317.[82–84] The investigation was extended to fluorescence quenching effects on model compounds **8** where an amide is rigidly held in



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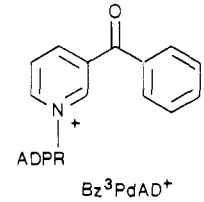
a fixed orientation with respect to an indole ring. It was found that in protic media fluorescence quenching occurs when the amide group is above the indole nucleus.[85]

Affect of Coenzyme Substitution on Substrate Specificity

The coenzyme analogue 3-benzoylpyridine–adenine dinucleotide (bz^3PdAD^+) **9** has been prepared earlier and among the hydrogenases tested, it was found to be active as a coenzyme only with LADH.[86] Indeed the



Bz^3PdAD^+

9

coenzyme binding site of LADH presents the unusual feature that the amide of the nicotinamide ring is located in the LADH–coenzyme–ligand complexes in a rather open space very close to the substrate binding site.[1] This induced the idea that the phenyl ring of bz^3PdAD^+ occupies partly the substrate binding site and this has been confirmed by a model study. Thus changes in the substrate specificity and possibly in the kinetic mechanism are expected.

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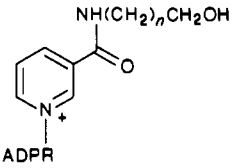
Primary alcohols, ethanol, 1-propanol, 1-butanol, and 1-pentanol are oxidized by bz^{3}PdAD$^+$ **9** in the presence of LADH, but no reaction is observed with 2- and 3-pentanol, isopropyl alcohol, or cinnamic alcohol. No chromophoric ternary complex LADH–bz^{3}PdADH–*p*-(dimethylamino)cinnamaldehyde is detected. The kinetic mechanism of ethanol oxidation and acetaldehyde reduction with LADH in the presence of 3-benzoyl dinucleotides was then studied. As expected the mechanism is rapid equilibrium random bi–bi. So the ethanol binding before the coenzyme binding becomes detectable, whereas with NAD$^+$, this represents only a minor pathway.[87]

Thus, LADH is a more substrate specific enzyme with 3-benzoyl dinucleotide. It would be interesting to test whether some molecule that has polar groups and does not react with NAD(H)$^+$ in the presence of LADH might prove to be a substrate with coenzyme analogues bearing an appropriate group at C-3 of the pyridinium ring capable of interacting with the polar group of the substrate.

This hypothesis agrees with the activity described in a preliminary manner for analogues where the alcohol function is covalently linked to the coenzyme–*N*-(ω-hydroxyalkyl)nicotinamide–adenine dinucleotide **10** where intramolecular redox reaction is observed.[88]



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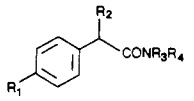
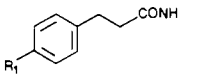
Design of Inhibitors from the Three Dimensional Structure of LADH

In recent years, as more information on the structure of receptor sites has become available, it has been possible to adopt a rational approach to the design of ligands instead of relying on structure–activity relationships.[89-93] The efficiency of this rational approach is illustrated by the modeling of inhibitors for LADH.[94] Of 57 compounds prepared, 16 shows an inhibition constant of 1 μM or below. In fact some poor inhibitors were designed specifically to demonstrate that they would be poor inhibitors. In the model it was assumed that the molecules bound to LADH–NADH would complex as ligands to the active-site zinc ion. The active site is rather closed so that N-substituted amides or acetamides could not be bound with coordination to the zinc ion. Indeed these compounds **14**, **15**, and **21** are rather poor inhibitors as shown in Table I.

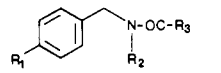
Table I



		K_i
11	$R_1 = R_2 = R_3 = R_4 = H$	140
12	$R_1 = R_3 = R_4 = H$, $R_2 = CH_3$	900
13	R_1 = butoxy, $R_2 = R_3 = R_4 = H$	7
14	R_1 = butoxy, $R_2 = R_4 = H$, $R_3 = CH_3$	200
15	R_1 = butoxy, $R_2 = H$, $R_3 = R_4 = CH_3$	1200
16	R_1 = -*O*-cyclohexyl, $R_2 = R_3 = R_4 = H$	1



		K_i
17	$R_1 = H$	25
18	R_1 = butoxy	0.12



		K_i
19	$R_1 = R_2 = R_3 = R_4$	3
20	R_1 = butoxy, $R_2 = R_3 = H$	0.04
21	$R_1 = R_2 = H$, $R_3 = CH_3$	1000
22	$R_1 = R_3 = H$, $R_2 = CH_3$	25
23	OHC–NH–$(CH_2)_{12}$–NH–CHO	0.058

The space in the hydrophobic substrate binding site around the phenyl ring of phenylacetamide is somewhat restricted. Introduction of a methylene group reduces this crowding and therefore 3-phenylpropionamide **17** is a better inhibitor. The para position of both amides is in an open space, and, as anticipated, substitution in this position increases the binding as shown for amides **13** and **18**. We thought that having a second hydrogen bond close to the zinc ion would increase the binding, and therefore we prepared the formamide derivatives. The oxygen is expected to coordinate to the zinc ion and the N–H to form a hydrogen bond to the hydroxyl group of Ser-48. As expected, formamide **19** is a better inhibitor than the amides **11** and **17**. Polar residues at the bottom of the substrate binding site could provide additional binding interactions. α,ω-Bifunctional compounds (e.g., **23** in Table I) are even better inhibitors. Not unexpectedly these inhibitors bind to the metal substituted LADH[95] and show activity in vivo by reduction of the ethanol metabolism rate.[96]

This work shows that structural knowledge of the receptor site is a powerful tool for the design of ligands to this site. A related approach has been taken to design active-site-directed reagents binding to LADH–NADH coenzyme and reacting with a methionine residue.[97,98]

Alcohol Dehydrogenase as a Redox Catalyst

The fact that there is a difference in the crystal forms of neat LADH and of LADH–coenzyme complex reflects a conformational change of the protein on binding the coenzyme. The enzyme crystals of LADH–coenzyme complex are active as shown by microspectro-

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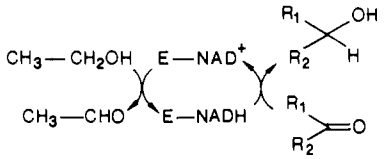
photometry[99] and the coenzyme is firmly bound. Taking this information into account, we prepared glutaraldehyde cross-linked crystals of the complex LADH–NADH–Me_2SO, in order to study the redox reaction. In the crystals the coenzyme is firmly bound and the redox activity is stable for a long time. Since the coenzyme does not dissociate, a redox system where an alcohol is oxidized while a carbonyl compound is



reduced, is set up.[100] The cross-linked crystalline enzyme is much more stable than the enzyme in solution towards organic solvents, and the stability is increased further by treatment of the cross-linked enzyme crystals with zinc salts. The cross linked LADH–coenzyme crystals are a convenient redox catalyst, and their use in a flow reactor can be envisioned.

This approach offers a new solution to the problem of coenzyme recycling, which is of great concern for the use of dehydrogenases as catalysts in organic chemistry.[101]

Concluding Remarks

We would like to emphasize two points. First it is a great benefit to crystallographers working with large biological molecules to interact not only with biologists but also with chemists who are able to design organic molecules as aids for attacking the structural problems. The second is that it is most useful for chemists to use the structure of these large biological molecules to relate their results to the structure and to conceive new questions.

We gratefully acknowledge the valuable contribution of the co-workers whose names appear on the articles quoted in this Account. Acknowledgements is also made to the Centre National de la Recherche Scientifique and to the Conseil Suédois de Recherches en Sciences Naturelles.

Registry No. LADH, 9031-72-5.

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CORRESPONDENCE

High Tech + Illiteracy = State-of-the-Art Science?

Contrary to popular belief more sophisticated equipment does not always help to generate better scientific results. As a journal editor and referee I am regularly confronted with experimental data where the compounds isolated are characterized by a *melting temperature* rather than a *melting range*. According to the usual convention this implies that the solids in question possess melting ranges of less than 0.5 °C. While this may indeed be so in rare cases of exceptional purity (typically requiring zone melting etc.) a more likely explanation would be that most of these data pertain to samples of ordinary purity (i.e., suitable for elemental analysis) from laboratories equipped with automatic melting point apparatus and manned by mediocre chemists.

The common types of automatic melting point apparatus record the temperature where the sample reaches a predetermined degree of transparency rather than the start and end temperatures of the melting process, the actual measure of a solid's fusion behavior short of a full differential thermal analysis. Thus, while automatic melting point apparatus is a boon wherever large series of uniform samples have to be processed, it can definitely not replace the good old-fashioned determination of the melting range under the microscope.

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