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Ligand discovery using the inter-ligand Overhauser effect: horse liver alcohol dehydrogenase

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Abstract

Two dimensional nuclear Overhauser effect spectroscopy (NOESY) studies on horse liver alcohol dehydrogenase (LADH) in the presence of several ligands revealed unanticipated cross peaks arising from inter-ligand Overhasuer effects (ILOEs) connecting resonances of an inhibitor, *m*-methylbenzamide, and the reducing agent, cyanoborohydride, initially present to maintain NADH in the reduced state. The presence of NADH was not required to observe of these inter-ligand Overhauser effects. A model for the ternary complex was developed in which the methylbenzamide inhibitors bind to the hydrophobic pocket of the active site involved in benzyl alcohol binding, while the cyanoborohydride coordinates directly with Zn^{2+} at the active site. The observation of these effects supports the use of inter-ligand Overhauser effects for the identification of unanticipated ternary complexes that are of potential utility for the development of novel enzyme inhibitors.

Abbreviations: ILOE, inter-ligand Overhauser effect; LADH, horse liver alcohol dehydrogenase; NOESY, nuclear Overhauser effect spectroscopy.

Introduction

Many enzymes have the ability to organize multiple ligands into geometrical arrangements which facilitate catalysis. For example, pyridine nucleotide-dependent oxido-reductases represent one of the most extensively studied class of such enzymes. In many instances, ternary complexes have been observed crystallographically or inferred kinetically which do not involve pairs of reactants, but arise from unusual and often unanticipated complexes involving buffers, metal ions, or other organic molecules that happened to be present in the media during kinetic studies or crystallization. As one example, assays of γ-glutamyl transpeptidase in borate buffer led to the discovery of a ternary enzyme-serine-borate complex (Revel & Ball 1959, London & Gabel 2001). Although in many cases such complexes have been identified from crystallographic analyses, NMR offers a potential alternative approach to the identification of such ternary complexes in solution. Probably the most generally useful approach for the identification of such complexes formed from weakly binding organic ligands, i.e. typically with millimolar to micromolar dissociation constants, is based on the transfer of interligand Overhauser effects (ILOEs) present in the ternary complex to the uncomplexed ligands (London 1999). Such effects can generally be observed at relatively high, millimolar ligand concentrations and their interpretation requires only the assignment of the ligand resonances. This phenomenon has previously been demonstrated in systems closely related to the functional ternary complexes of several enzymes (Barsukov *et al.* 1996, Li *et al.* 1999, 2001). However, this organizational behavior of biological macromolecules also can be utilized for the development of novel ligands and pharmacologi-

cally useful inhibitors, and hence is of potential value for biopharmacological exploitation. In the present study, we demonstrate that such effects can be observed from unanticipated ligand binding to alcohol dehydrogenase.

Materials and methods

Horse liver alcohol dehydrogenase (LADH) was obtained from Boehringer Mannheim (Germany) and prepared for NMR studies by dialysis into a D_2O buffer containing 20 mM phosphate, pH 7.2. NADH was obtained from Sigma, and all other chemicals were obtained from Aldrich.

All NMR measurements were performed at 500 MHz on a Varian Unity*Plus* 500 spectrometer at 20 °C using a Nalorac Probe (Martinez, CA). Phase-sensitive nuclear Overhauser effect spectroscopy (NOESY) spectra were acquired consisting of 1024 real t_2 points and 256 complex t_1 points with acquisition times of 85 ms (t_2) and 21 ms (t_1). Thirty-two scans were acquired per t_1 increment to allow complete phase cycling for suppression of transverse magnetization during the mixing time, axial peaks, and F_2 quadrature images. F_1 quadrature detection was achieved via the States-TPPI method (Marion *et al.* 1989). The residual water proton signal was suppressed by pre-saturation for 0.5 s at 0 dB power (although some residual intensity is apparent in the spectrum obtained at $\tau_m = 500$ ms). The total repetition time for each scan was 2.1 s, corresponding to an average accumulation time of 15 h. The spectra were processed using Felix 97 (MSI, San Diego, CA) with 90° shifted squared sinebell window function in both dimensions and baseline correction in the t_2 dimension. After zero-filling of the t_1 dimension, the final matrix contained 1024 × 1204 data points prior to Fourier transformation.

Results and discussion

As part of a series of studies of enzymes involved in hydride transfer reactions, we recently investigated the possibility of interligand Overhauser effects for a series of complexes with horse liver alcohol dehydrogenase (LADH). In one set of studies, we examined the ternary complex that has been reported to form between LADH, NADH and *m*-methylbenzamide. The latter has been reported to be a weak inhibitor of LADH with a $K_I = 33$ μM (Sigman 1967, Sarma & Woronick 1972). Since there is a tendency for reduced pyridine nucleotides to auto-oxidize and since the experiments typically last for at least several hours, sodium cyanoborohydride, a mild reducing agent which has been reported to reduce NAD^+ to NADH (Avigad 1979), was added to the system in order to maintain the NADH in the reduced form. Although it had been suggested that in the ternary methylbenzamide-NADH-LADH system the benzamide ring might stack in a plane parallel to the dihydronicotinamide (Sarma & Woronick 1972), no inter-ligand Overhauser effects connecting these ligands were observed. The binding of both ligands to the enzyme was supported by the observation of negative intra-ligand transferred NOE peaks. Transferred NOE resonances have been observed previously in studies of the NAD-LADH binary complex (Gronenborn & Clore 1982, Andersen *et al.* 1987). However, the observed transferred NOE peaks for the NADH in this study were considerably weaker than those observed for the methylbenzamide.

The absence of ILOE resonances connecting the two ligands can be explained on the basis of a structure in which the protons on the bound NADH are relatively distant from those on the methylbenzamide. However, the exchange kinetics for NADH are fairly slow; a dissociation rate constant of 5.2 s^{-1} has been reported for the NADH-LADH complex (Kamlay & Shore 1983), and the rate might be even slower for the ternary complex. The slower kinetic process would also explain the weaker transferred NOE resonance intensities for the NADH. Hence, the slow dissociation rate constant for the NADH-LADH complex is probably also a factor in explaining the absence of methylbenzamide-NADH ILOE resonances.

In these spectra, a set of proton resonances of the BH_3 group of the cyanoborohydride, also present at a 5 mM concentration, was observed at $\delta = 0.23$ ppm and exhibited splitting due to the $^1J(^{11}B-^1H) = 91.3$ Hz. Unexpectedly, the NOESY spectra revealed a set of interligand Overhauser cross peaks connecting the BH_3 protons of the cyanoborohydride with the proton resonances of the inhibitor, *m*-methylbenzamide (Figure 1). The negative sign of the resonances (in phase with the diagonal) indicates that these cross peaks arise from a complex with a long rotational correlation time. Involvement of the enzyme was confirmed by the observation that no ILOE cross peaks were observed in solutions containing 5 mM *m*-methylbenzamide, 5 mM $NCBH_3$, but no alcohol

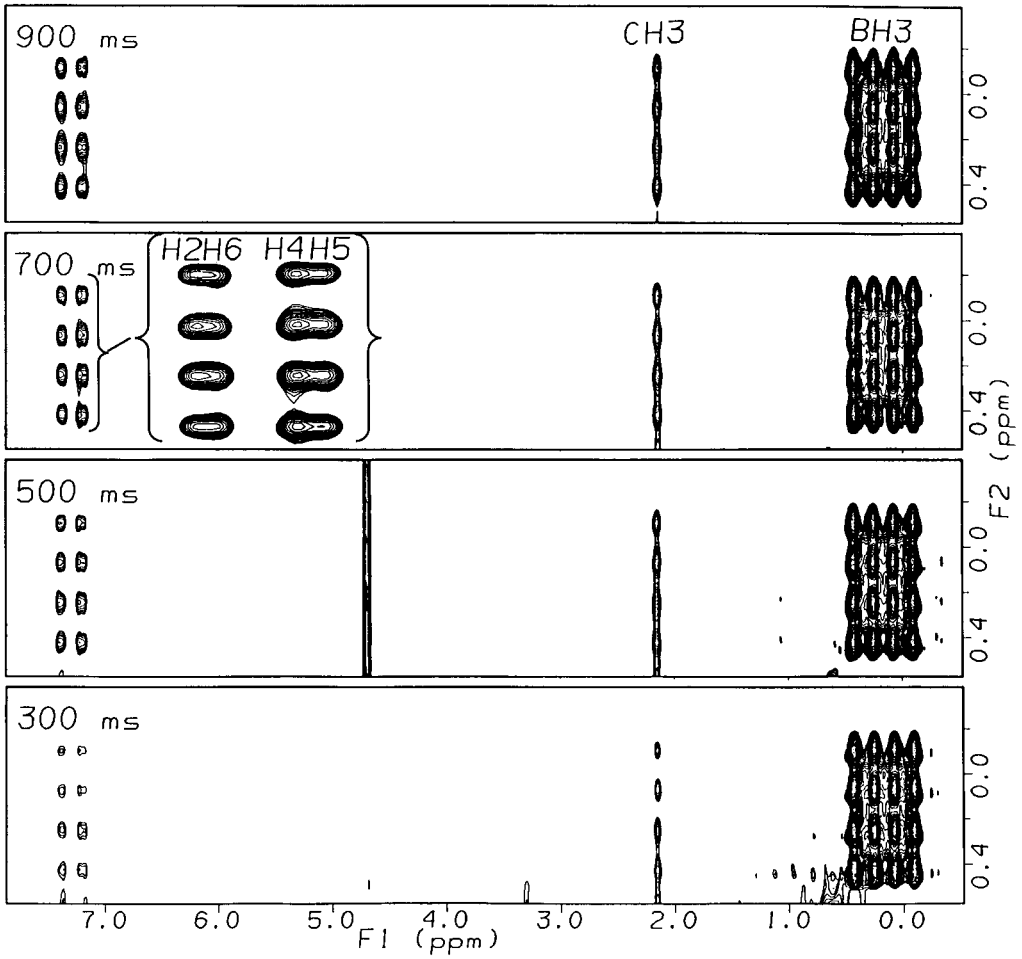

Fig. 1. Strips of 2D ^1H $-^1H$ NOESY spectra of a sample containing 5 mM sodium cyanoborohydride, 5 mM *m*-methylbenzamide, 0.2 mM LADH in 20 mM phosphate (pH 7.2) buffer in D_2O, for the mixing times indicated in the upper left of each spectrum. The spectra show the BH_3 resonances on the diagonal and the cross peaks with the methyl and aromatic protons of the *m*-methylbenzamide for the mixing times indicated in the upper left of each spectrum. The proton resonances of the BH_3 are split due to the $^1J(^{11}B$ $-^1H)$ scalar coupling. The assignments of the four aromatic protons are given in the inset on the τ_m = 700 ms spectrum. The enzyme was prepared by dialysis horse liver alcohol dehydrogenase (Boehringer Mannheim, Germany) into a D_2O buffer containing 20 mM phosphate at pH = 7.2 (meter reading). Phase sensitive NOESY spectra were acquired consisting of 1024 real t_2 points and 256 complex t_1 points with acquisition times of 85 ms (t_2) and 21 ms (t_1). Thirty-two scans were acquired per t_1 increment to allow complete phase cycling for suppression of transverse magnetization during the mixing time, axial peaks, and F_2 quadrature images. F_1 quadrature detection was achieved via the hypercomplex method. The residual water proton signal was suppressed by pre-saturation for 0.5 s at 0 dB power (although some residual intensity is apparent in the spectrum obtained at τ_m = 500 ms). The total repetition time for each scan is 2.1 s, corresponding to an average accumulation time of 15 h. The spectra were processed using Felix 97 (MSI, San Diego, CA) with 90° shift squared sinebell window function in both dimensions and baseline correction in the t_2 dimension. The final matrix contains 1024 × 1204 data points with zero-filing of the t_1 dimension to 1024 data point prior to Fourier transformation.

dehydrogenase. Hence, these observations indicate the formation of a ternary $NCBH_3$-*m*-methylbenzamide-LADH complex in which the two ligands are in close proximity.

The dependence of these cross peaks on the presence of NADH was further evaluated, and it was found that omission of the NADH from the sample had no significant effect on the observed ILOE peaks. Subsequent studies using *p*-methylbenzamide (K_I = 54 μM) (Sarma & Woronick 1972) and *o*-methylbenzamide (no K_I reported) indicated that both inhibitors produced analogous cross peaks (Figure 2). The relative ILOE intensity pattern observed at shorter mixing times indicates that the BH_3 group is close to the methyl groups of the methylbenzamides. The aromatic protons ortho to the methyl groups also give

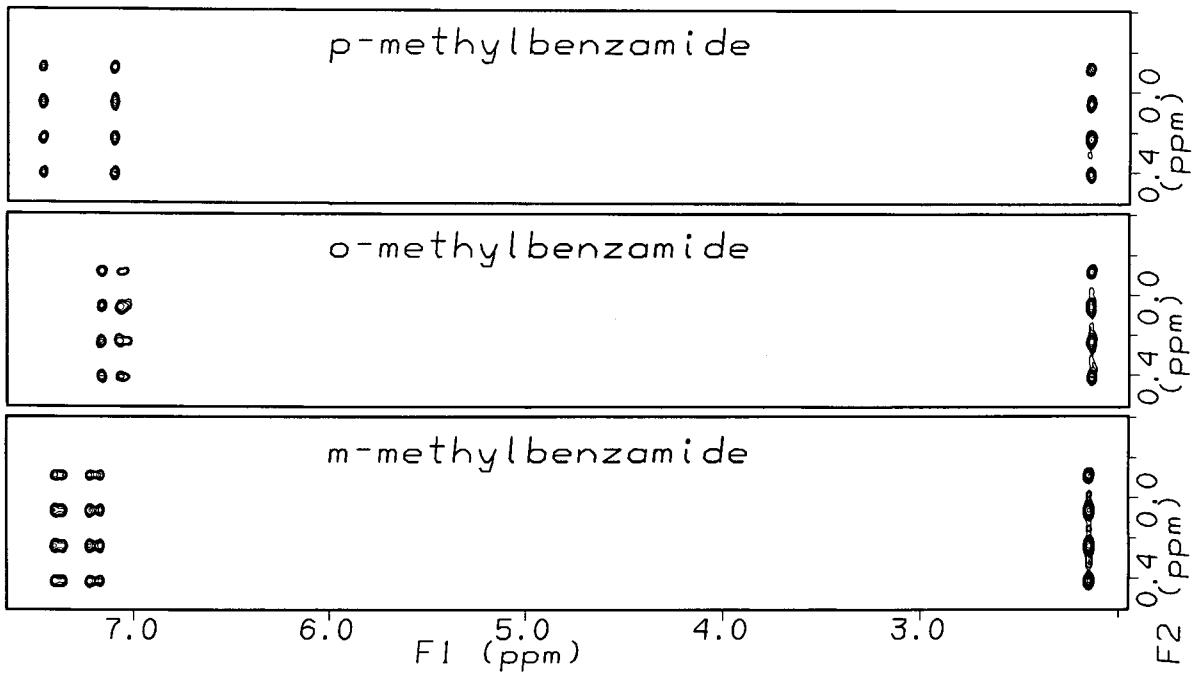

Fig. 2. Off diagonal region of the 2D ^{1}H $-^{1}H$ NOESY spectra (all at 700 ms mixing time) of samples containing 5 mM *para*, *ortho* or *meta* methylbenzamide as indicated at the top of each spectrum. Samples also contained 0.2 mM horse liver alcohol dehydrogenase + 5 mM cyanoborohydride. The cross peaks are ILOEs between the BH_3 protons of cyanoborohydride and the methyl and aromatic protons of the methylbenzamides. As in Figure 1, cross peaks with the BH_3 appear as sets of four resonances corresponding to the z-spin states of the ^{11}B. Other NMR parameters as in Figure 1.

the most significant ILOE peaks (Figures 1 and 2), undoubtedly arising from a combination of direct and indirect relaxation pathways via the methyl protons.

Upon further consideration of the basis for these observations, the structural similarity between cyanoborohydride and ethanol led to the hypothesis that the cyanoborohydride might bind to the alcohol binding site of LADH, with the nitrogen directly coordinating Zn^{2+} at the active site. Such an interaction could be considered analogous to the binding of the alcohol oxygen to the Zn^{2+} in the catalytic complex (Eklund *et al.* 1982, Ramaswamy *et al.* 1994). Consistent with this hypothesis, previous studies of the effects of ions on modification of LADH by iodoacetate have supported the conclusion that cyanide binds to the catalytic zinc ion (Syvertsen & McKinley-McKee 1984). Interestingly, zinc-cyanoborohydride has been described as a modified reducing agent (Kim *et al.* 1985). However, if this hypothesis is correct, both the methylbenzamide inhibitor and the ethanol analog (cyanoborohydride) must form a complex at the active site simultaneously.

To further explore the ethanol/cyanoborohydride binding analogy, NOE studies of 5 mM *m*-methylbenzamide + 5 mM ethanol were performed in the presence of 0.2 mM LADH. In these studies, weak ILOE peaks (in comparison with those observed to the BH_3 of cyanoborohydride) are observed between ethanol and *m*-methylbenzamide protons (Figure 3). In contrast to this result, ILOE peaks connecting BH_3CN with ethanol were not observed if NADH was added to the above system. Hence, these data demonstrate that *m*-methylbenzamide and ethanol can form a ternary complex with LADH, but that a quaternary complex involving NADH, *m*-methylbenzamide, ethanol, and LADH does not appear to form. Thus, although the presence of methylbenzamide may reduce the affinity of the enzyme for ethanol, it apparently does not prevent binding. The basis for *m*-methylbenzamide inhibition of LADH therefore appears not to be a simple competition with ethanol.

As a model for the observed ternary complex, we have considered the structures reported for the complexes formed by LADH with substituted benzyl alcohols (Eklund *et al.* 1982, Ramaswamy *et al.* 1994). For these ligands the interaction with the active site of the enzyme involves coordination of the alcohol oxygen to the zinc, hydrogen bonding of the hydroxyl

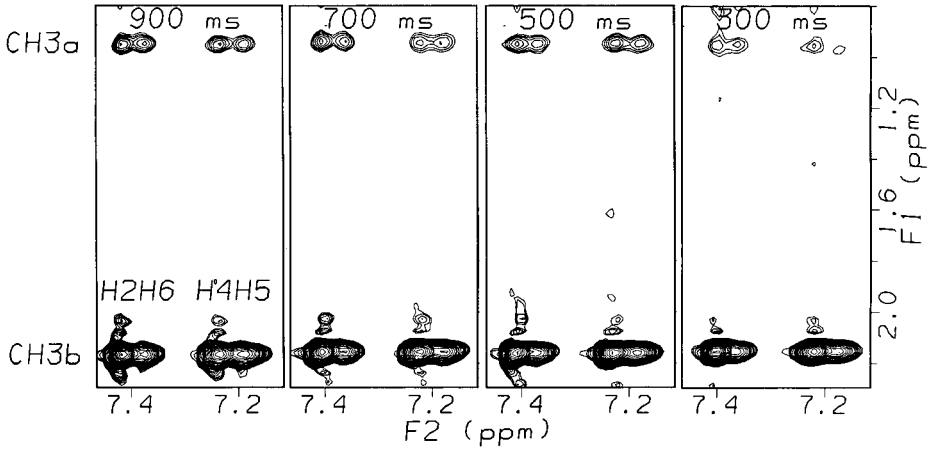

Fig. 3. Portion of the NOESY spectrum obtained on a sample containing 0.2 mM LADH, 5 mM *m*-methylbenzamide, and 5 mM ethanol. Other conditions as in Figure 1. The strong intramolecular transferred NOE cross peaks connecting the H2, H6, H4, and H5 protons of the *m*-methylbenzamide with the C-3 methyl group ('CH3b') (at $\delta = 2.14$ ppm) and the weaker ILOE cross peaks connecting these protons with the methyl protons of ethanol ('CH3a', $\delta = 0.95$ ppm) are readily observed. The spectra corresponded to mixing times of 300, 500, 700, and 900 ms, as indicated. The intensity pattern of the ILOE resonances is H2 > H4–H6 > H5.

proton to Ser-48, and interaction of the aromatic ring with a hydrophobic pocket involving residues Leu-57, Phe-93, Leu-116, Phe-140, Leu-141, Val-294, Leu-309 and Ile-318 (Figure 4A). Thus, it seems possible that, as long as the steric interference can be minimized, the cyanoborohydride can coordinate to the active site zinc ion, while the methyl benzamide is bound in the hydrophobic pocket that accommodates the benzyl alcohols, and may also hydrogen bond to Ser-48 (Figure 4B). The structure of the proposed ternary complex is consistent with the relative intensities of the observed ILOE peaks which for the *o*- and *m*-methyl benzamides show the greatest intensity with the methyl cross peak, and then to the protons located *ortho* to the methyl groups (Figure 1).

The model shown in Figure 4B is also consistent with the results of ^{113}Cd NMR studies of the cadmium-substituted LADH (Bobsein & Myers 1981). For the cadmium substituted enzyme, trifluoroethanol or pyrazole, inhibitors which subsequently have been shown to directly bind to the Zn^{2+} in the active site (Bahnson *et al.* 1997, Becker & Roberts 1984), produced ^{113}Cd shifts of 75 ppm in the LADH-NAD^+-prazole ternary complex. In contrast, a weaker inhibitor containing an amide group, *n*-butyramide, produced only a small ^{113}Cd shift of 3 ppm. This result indicates significant differences between the binding of the butyramide and pyrazole inhibitors, most probably involving a lack of direct coordination of the amide ligand to the Zn^{2+}/Cd^{2+} at the active site. Hence, this analysis suggests that the binding of the cyanoborohydride and the methyl benzamide would not compete for coordination with the active site Zn^{2+}, consistent with the structure shown in Figure 4B.

In order to further evaluate this model, we performed a competition experiment in which 18 mM *p*-fluorobenzyl alcohol was added to the sample containing 5 mM *p*-methylbenzamide, 5mM cyanoborohydride, and 0.2 mM LADH. Addition of the *p*-fluorobenzyl alcohol eliminated the ILOE peaks connecting the BH_3 and the protons of the *p*-methylbenzamide. Unexpectedly, cross-peaks were observed between the BH_3 of the cyanoborohydride and the aromatic protons of the *p*-fluorobenzyl alcohol with the greatest intensity to the protons *ortho* to the fluorine. These ILOE data provide support for the competitive binding of fluorobenzyl alcohol and methylbenzamide, as postulated above. The results confirm that the interaction of the methyl benzamide with the cyanoborohydride results from binding of the former to the portion of the active site involved in binding benzyl alcohol. However, the observation of the ILOEs connecting *p*-fluorobenzyl alcohol with the cyanoborohydride apparently indicates either that both the benzyl alcohol and the cyanoborohydride can complex to the Zn^{2+} simultaneously, or that there may be a binding mode for the benzyl alcohol in this complex which does not involve direct coordination with the active site Zn^{2+}, or finally that in this complex, the cyanoborohydride may bind in a configuration different from that shown in Figure 4B. The possibility of having two ligands interacting directly with the Zn^{2+}

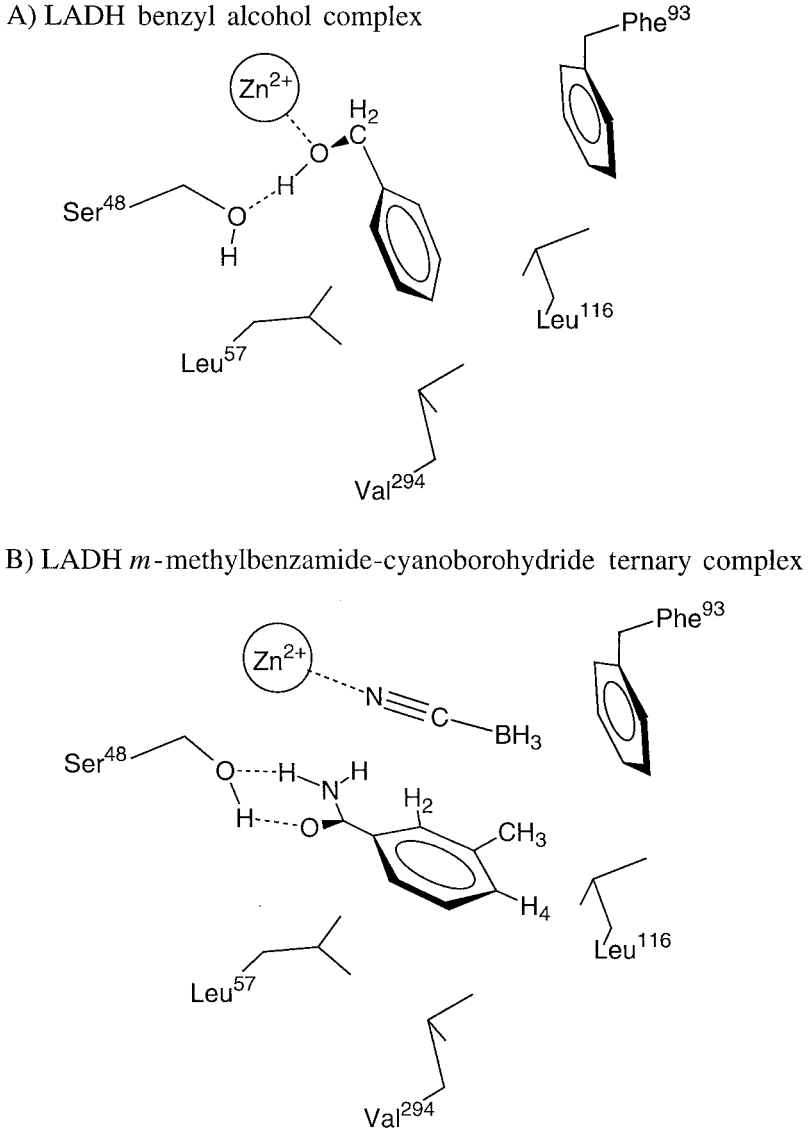

Fig. 4. Schematic representation of the active site of LADH showing (A) the complex formed with substituted benzyl alcohols based on Eklund *et al.* (1982) and Ramaswamy *et al.* (1994); and (B) the proposed ternary complex with *m*-methylbenzamide and cyanoborohydride.

is suggested by previous studies indicating formation of a (bidentate) complex of 1,10-phenanthroline with LADH (Sartorius *et al.* 1988, Wang *et al.* 2000).

The LADH system has been extensively studied and many strong inhibitors have been identified (Theorell *et al.* 1969, Schindler *et al.* 1998, Ramaswamy *et al.* 1997, Cho *et al.* 1997). The present results demonstrate, however, that new and unexpected binding relationships among LADH ligands are readily identified based on the observation of interligand Overhauser effects. Such studies can provide new insight into the structure and biochemistry of the active site and are of potential use for the design of new enzyme inhibitors, of potential value in the pharmaceutical industry.

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