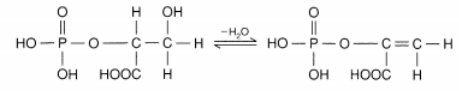
2.10: Nucleophilic Addition of O H − OH− and H − H−

Nucleophilic addition of OH^- ions as a step in enzymatic pathways is not restricted to hydrolytic processes; it often occurs in lyases, the class of enzymes catalyzing removal (or incorporation in the reverse reaction) of neutral molecules such as H_2O—but also NH_3, CO_2, etc.—from a substrate. It is outside the scope of this section to review all other mechanisms involved in lyase reactions, especially because they are not reducible to common steps and because several of them do not require the presence of a metal ion. We restrict ourselves to H_{2O} removal (or incorporation), a widespread feature of which seems to be the splitting of water into the constituents H^+ and OH^- ions at some step of the mechanism. As an example, the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate catalyzed by enolase, a Mg-activated enzyme,



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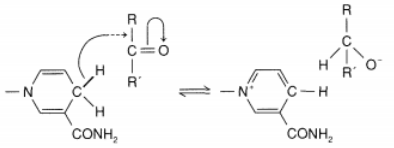
has been shown by kinetic isotope-effect studies[150] to proceed via fast H^+ removal from substrate followed by slow release of the product, and finally by release of OH^-. The role of a metal ion like magnesium might be to activate the substrate by coordinating the phosphate group, rather than by providing a coordinated hydroxide for nucleophilic attack.

Other lyases, however, contain transition metal ions [often iron(II)], and their main role might well be that of lowering the pK_a of water. None of them, however, is yet known well enough to allow a detailed discussion of the molecular mechanism. A striking exception is carbonic anhydrase, which has been so extensively and successfully studied that it is ideal as a case study (Section IV).

Hydride transfer is another elementary process encountered in many enzymatic reactions. Although hydride transfer implies a redox reaction, it also involves nucleophilic attack on substrate as in the foregoing examples. Unlike OH^-, hydride ions do not exist in aqueous solutions as free ions. In biological systems hydride is always directly transferred from one organic moiety to another by simultaneous breakage and formation of covalent bonds. The activation energy for this process is much higher than, for example, that of H^+ transfer via the formation of hydrogen bonds. Moreover, unlike hydrogen-bonded species, there is no intermediate in the process that can be stabilized by the catalyst. Instead, reacting species can be destabilized in order to lower the activation energy barrier. The role of the enzyme, and of the metal ion when present, is to provide binding sites for both substrates. The enzyme achieves this both geometrically, by allowing for proper orientation of the groups, and electronically, by providing energy to overcome the activation barrier.

These general concepts can be exemplified by liver alcohol dehydrogenases (LADH), dimeric zinc enzymes of 80 kDa that catalyze the following class of reactions using the NADH/NAD^+ system as coenzyme (or, really, as cosubstrate):



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In particular, LADHs catalyze the reversible dehydrogenation of primary and secondary alcohols to aldehydes and ketones, respectively. Other enzymatic activities of LADHs are aldehyde dismutation and aldehyde oxidation.[151] The physiological role, although surely related to the metabolism of the above species, is not definitely settled. Much effort is being devoted to understanding the mechanism of action of this class of enzymes, which have obvious implications for the social problem of alcoholism.

Each monomer unit of LADH contains two zinc ions: one coordinated to four cysteine sulfurs, the other coordinated to two cysteine sulfurs, one histidine nitrogen, and a water molecule. The former has no apparent role in catalysis; the latter is essential for catalytic activity. The x-ray structure of the metal-depleted enzyme from horse liver has been solved at 2.4 Å resolution, and that of the holoenzyme at 2.9 Å resolution (Figure 2.34 See color plate section, page C-6.). Many crystal structures are also available for binary complexes with substrates, pseudosubstrates, or coenzymes, as well as for ternary complexes with coenzyme and substrates.[152] The very detailed picture emerging from such structural information has helped us understand how LADH functions. As will be evident from the following discussion, elucidation of this mechanism also reveals some important fundamental chemistry.

A key property of the enzyme, established by x-ray data, is the existence of two protein domains in each monomer that are relatively free to rotate relative to each other. The apo- and holo-enzymes exist in the so-called open form, whereas binding of NADH coenzyme induces rotation of one domain, resulting in the so-called closed form[153,154] (Figures 2.34 and 2.35). Closure brings the catalytic zinc ion into an ideal position to bind the aldehyde substrate in such a way that the reactive CH_2 group of the nicotinamide ring of NADH points toward the carbonyl carbon (Figure 2.35).

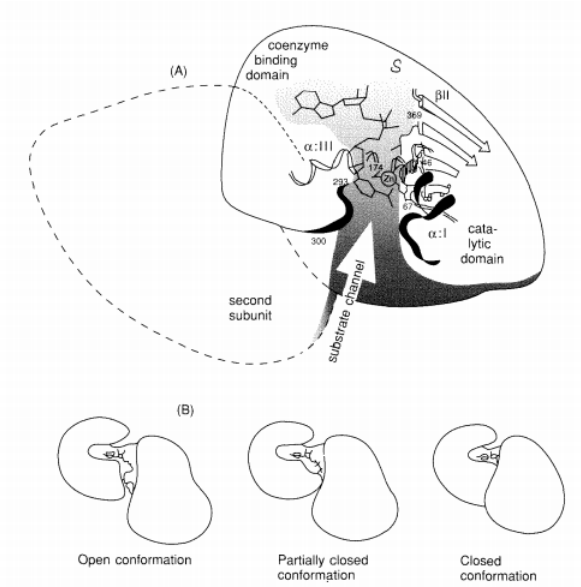
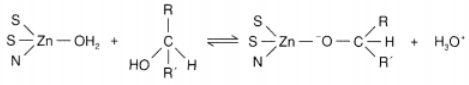


Figure 2.35 - Schematic drawing of (A) the LADH dimer and (B) the domains constituting the active site of a subunit.[154]

The main functions of the metal are thus to orient the substrate geometrically and to polarize the carbon-oxygen bond. Although the latter makes obvious chemical sense for the aldehyde reduction reaction, since polarization of the C=O bond facilitates nucleophilic attack of hydride at the carbonyl carbon, coordination of an alcohol to a metal is expected to decrease the alcohol's tendency to transfer hydride to NAD^+, unless the hydroxyl proton is released upon coordination.[155]



(2.23)

Formation of an alkoxide ion as an intermediate has often been questioned, because the pK_a of the alcohol would have to be reduced by about 10 units upon coordination.[156] The possibility that hydride transfer from alcohol to NAD^+ and hydroxyl proton release could occur simultaneously is attractive, but careful experiments have shown that the two steps must be kinetically separate.[157] We summarize here the key information that leads to a full, although circumstantial, rationalization of the chemical behavior of the enzyme.

1. The activity versus pH profiles[156,158] are bell-shaped, with k_{cat} increasing with a pK_a below 7, reaching a plateau, and decreasing with a pK_a above 11, and K_m increasing with a pK_a of about 9.
2. X-ray data show that the zinc ion is accessible to solvent in the open conformation, much less so in the closed conformation when the reduced coenzyme is bound, and inaccessible when the substrate is coordinated to the metal in the ternary complex, extruding all the water molecules from the active site.[152] None of the complexes has a coordinated water molecule as a fifth ligand when substrates or inhibitors are bound to the metal. The metal ion is always four coordinate and pseudotetrahedral. Computer graphics reveal beyond any doubt that there is no room for a fifth ligand in the active site, at least in the closed form.
3. Many (although not all) spectroscopic data on metal-substituted derivatives and their binary and ternary complexes have also been interpreted as indicative of a four-coordinate metal.[159] Even nickel(II) and copper(II), which have little tendency to adapt to a pseudotetrahedral ligand environment, do so in LADH, the electronic structure of the latter resembling that of blue proteins (Figure 2.36).[160]

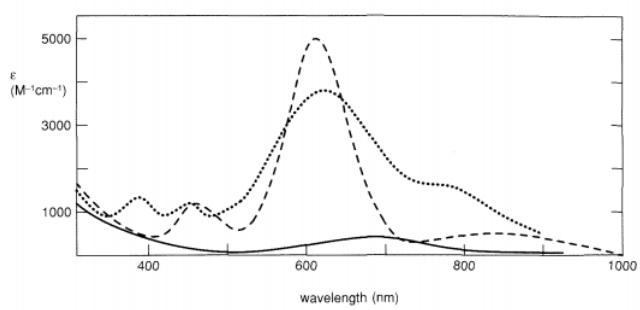
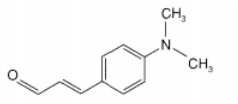


Figure 2.36 - Electronic spectra of liver alcohol dehydrogenase substituted with copper at the catalytic site (• • • • •),[160] *together with the spectra of blue (stellacyanin, ---)*[161] *and non-blue (superoxide dismutase, ———)*[162] *copper proteins.*

4. The substrate binding site is actually "created" by the closure of the protein (Figure 2.34). The reactive species are thus trapped in an absolutely anhydrous environment. The chromophoric aldehyde DACA has been extensively used as an "indicator" of the polarity of the binding site. Large red shifts of the ligand π-π* transition upon binding indicate the polarity of the site to be much higher than in water; there is a further sizeable increase in polarity when NAD^+ instead of NADH is bound in the ternary complex.[163]



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5. The electronic spectra of the cobalt-substituted derivative are characteristically different when different anions are bound to the metal (Figure 2.37).[164] A catalytically competent ternary complex intermediate displays the electronic absorption pattern typical of anion adducts.[166]

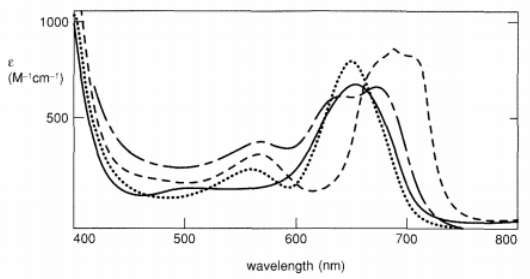

Figure 2.37 - Electronic spectra of liver alcohol dehydrogenase substituted with cobalt at the catalytic site. Binary complex with NAD^+ (———);[165] *ternary complex with* NAD^+ *and* Cl^-(---);[165] *binary complex with acetate* (• • • • •);[164] *intermediate in the oxidation of benzyl alcohol with* NAD^+ (— — — —).[166]

6. From extended kinetics measurements a protonation scheme (Figure 2.38) has been proposed that accounts for the many pK_a values observed under different conditions.[167] This scheme again requires formation of a coordinated alkoxide intermediate, but has the advantage of rationalizing in a simple way a complex pattern. In essence, the only relevant acid-base group supplied by the enzyme is the metal-coordinated water, which has a pK_a of 9.2 in the free enzyme (open form). Upon binding of NADH the pK_a increases to 11.2. Since NADH dissociation is the last and rate-limiting step of the alcohol oxidation reaction, the decrease in k_{cat} with this pK_a is accounted for by a decrease in dissociation rate of NADH from the hydroxo form. On the other hand, the pK_a of water is decreased to 7.6 upon binding of NAD^+. These rather large changes in both directions are best explained by a marked sensitivity of the coordinated water molecule to the polarity of the environment, which, with the possible exception of the unligated form that has a more or less "regular" pK_a value of 9.2, can be almost completely anhydrous and much different from that of bulk water. The nonpolar nicotinamide ring of NADH decreases the overall electrostatic interactions of the water molecule, whereas the positive charge of NAD^+ drastically increases them. In this scheme, the association rates of both coenzymes are predicted to (and, in fact, do) decrease with a pK_a of 9.2, the dissociation rate of NAD^+ is predicted to (and does) decrease with a pK_a of 7.6, and the dissociation rate of NADH is predicted to decrease with a pK_a of 11.2 (and, indeed, it is pH-independent up to and above pH 10).

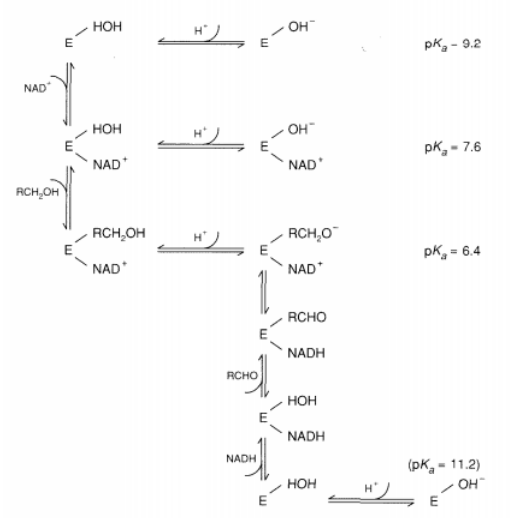

Figure 2.38 - Protonation scheme for LADH and its adducts with coenzymes and substrates.[157,167]

The decrease of k_{cat} at low pH depends on an ionization that in turn depends on the substrate. This pK_a must be that of the coordinated alcohol; at too Iowa pH, deprotonation of the coordinated alcohol becomes the rate-limiting step. The pK_a values observed for this process range from 6.4 for ethanol to 4.3 for triftuoroethanol. What is surprising for aqueous-solution chemistry—that the pK_a of a coordinated alcohol is lower than the pK_a of a coordinated water molecule—can now be explained in terms of the different polarity of the two adducts in LADH. In the binary complex with NAD^+ (pK_a = 7.6), the water molecule is still free to interact through H-bonding with the solvent and partially dissipate the electrostatic charge. In the ternary complex with any alcohol, the R group may prevent access of the solvent to the cavity, decreasing the dielectric constant of the medium. As a consequence, the polarity of the environment is increased. It is interesting to speculate that Nature could have chosen a stronger Lewis acid than a zinc ion coordinated to two negatively charged residues to decrease the pK_a of a coordinated alkoxide, but then the pK_a of the coordinated water would have simultaneously undergone a parallel and possibly even stronger decrease. Instead, LADH provides a self-regulating environment that is tailored to decrease the pK_a of a coordinated alcohol, once properly positioned, more than that of a coordinated water. The full catalytic cycle for the dehydrogenation reaction at pH around 7 can be summarized as follows (Figure 2.39):

1. NAD^+ binds to the open, water-containing form of the enzyme with a maximal on-rate. The pK_a of water is decreased to 7.6, but water is still mostly unionized.
2. A neutral alcohol molecule enters the crevice between the two domains, and coordinates the zinc ion by displacing the water molecule. The protein is still in the open form.
3. Domain rotation brings the protein into the closed form, excluding all the residual water molecules from the active site; the combined effect of the metal positive charge and of the unshielded positive charge of the nicotinamide ring lowers the pK_a of the coordinated alcohol below 7. A proton is expelled from the cavity, possibly via a hydrogen-bond network of protein residues.
4. Direct hydride transfer takes place from the alcohol CH to the 4-position of the properly oriented nicotinamide ring. The resulting ternary complex is an NADH-aldehyde adduct. The polarity of the active site dramatically drops.
5. The aldehyde product leaves and is replaced by a neutral water molecule (its pK_a now being 11.2). Additional water molecules can now enter the crevice, favoring the partial opening of the structure.
6. The loss of contacts between the two halves of the channel favors a complete opening and then the release of NADH, whose dissociation rate is maximal and pH-independent.

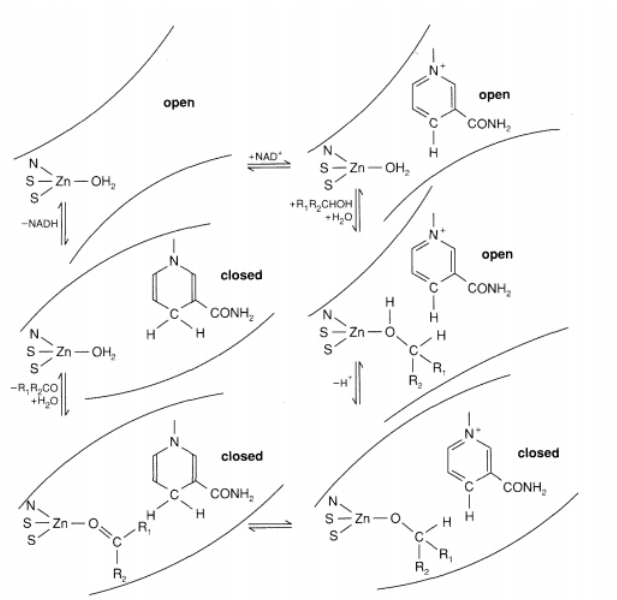

Figure 2.39 - Possible catalytic cycle of LADH.
