ON THE MECHANISM OF THE ETHANOL–DIPHOSPHOPYRIDINE NUCLEOTIDE REACTION

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SUMMARY

The explanation for the shift of the maximum of DPNH from 3400 Å to 3250 Å in the complex with alcohol dehydrogenase is derived from a consideration of environmental effects on the spectra of 1,4-dihydronicotinamides. It is concluded that a positively charged nitrogen in the form of an ammonium ion is located 3 Å from the nitrogen of the dihydropyridine ring and produces the shift by repulsion of the increased charge produced on the dihydropyridine nitrogen by light absorption. It is suggested that an ε-amino group of lysine provides the ammonium ion and a transition state for the reduction of acetaldehyde by DPNH is written utilizing this suggestion and some other conclusions from the literature.

INTRODUCTION

One of the important aims of studies of enzyme mechanisms is the description of the transition state (or states) involved in a given transformation. The usual technique for constructing such descriptions is to combine information on the nature of the groups present at the "active site" with principles derived from investigation of model systems. The purpose of the present note is to indicate how an important detail may be deduced for the "active site" of alcohol dehydrogenase from spectroscopic data on enzyme–DPNH complexes. In connection with previous ideas about "hydrophobic" regions at the "active site" of alcohol dehydrogenase[1], it is possible to write a reasonable mechanistic description of the ethanol-acetaldehyde transformation. Of the several other schemes for DPN action[2,3], only those which implicate the zinc ion as an integral part of the "active site" will be discussed[4,5].

DISCUSSION

CILENTO and his coworkers have reported that 1-benzyl-1,4-dihydronicotinamide has a dipole moment of 3.89 D and an ultraviolet absorption maximum which varies in position with the polarity of the solvent[6] (Table I). The charge rearrangement suggested by the conjugated system and the direction of the solvent effect is depicted by the formulae in Fig. 1.

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The complex of alcohol dehydrogenase and DPNH has an absorption maximum at 3250 Å in contrast to DPNH with a maximum at 3400 Å[7]. This observation has been repeated and extended to DPNH analogues and DPN-addition products[8]. The spectrum of the complex is very similar in intensity and shape to that of DPNH alone, suggesting that the *same electronic transition* is involved. Why, then, is the position of the maximum changed in the complex?

TABLE I

ULTRAVIOLET ABSORPTION OF 1-BENZYL-1,4-DIHYDRONICOTINAMIDE

Solvent	λ_{max}, *A*	E_T*
Diethyl ether	3400	84.1
Cyclohexane	3450	82.8
Ethanol	3540	80.7.
Water	3570	80.1

* Transition energies in kcal/mole.

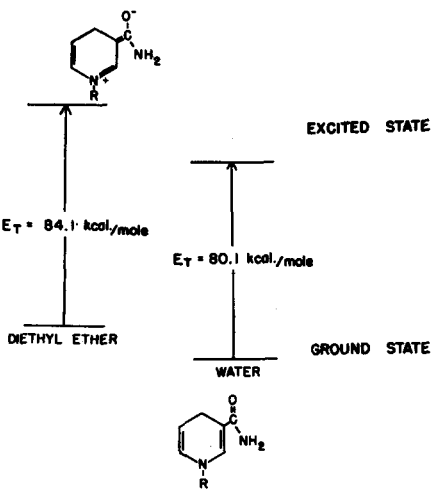


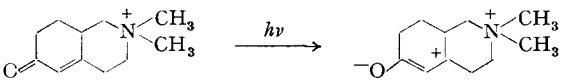
Fig. 1.

On the basis of the data for the model compound in Table I, we might conclude that the "active site" is non-polar in character, and this suggestion has been advanced[9]. It is, of course, an assumption that the relationship of the DPNH molecule to the enzyme is the same in the presence as in the absence of acetaldehyde. However, the solvent sensitivity of the light absorption of dihydronicotinamide is not large (*cf.* Table I), so that the "active site" would have to be hydrocarbon in nature to account for the shift found for the complex. It seems unreasonable to expect that the charged pyridinium ring of DPN would be generated in a non-polar environment; further, the specific hydrophobic character of a *portion* of the active site[1] would be lost.

The analogies to the Meerwein-Ponndorf-Oppenauer equilibrium which propose that the zinc ion play a vital role in DPN–DPNH reactions[4,5] require the metal ion to be located at the amide end of the dihydropyridine ring of DPNH. If a positively

charged species like the zinc ion were near the amide group, the electronic transition would be *lowered*, not raised in energy, since charge migrates in the transition towards the amide end of the ring. Even if there were not certain difficulties posed by the necessity for replacing coordinated water by the dihydro-pyridine ring, the spectroscopic data for the DPNH–enzyme complex do not favor a mechanism involving the zinc ion. Experiments with complexing agents[10] and competition with cadmium[11] imply that zinc serves to bind DPNH to the enzyme, probably through the pyrophosphate group. The interesting suggestion[12] that the stereospecificity of the hydride transfer promoted by alcohol dehydrogenase is associated with the zinc ion site is not in accord with the foregoing conclusion, namely, that the zinc ion is a moderate distance from the site at which the hydrogen transfer occurs.

A possible explanation for the shift in the spectrum of DPNH when complexed with an enzyme is suggested by data and calculations on an α, β-unsaturated ketone which possesses a positive charge in the form of a quaternary nitrogen near the β-carbon of the conjugated system[13]. In this case, a shift to shorter wavelengths (higher transition energies) is observed in the ketone containing the quaternary nitrogen as compared with that which had only a methylene group at the same position. Calculations by a method given in the literature[14] supported the idea that the change in maximum was due to an electrostatic effect, specifically the repulsion between the positive nitrogen and the increased positive charge at the β-carbon of the unsaturated ketone resulting from the electronic transition (Eqn. 1).



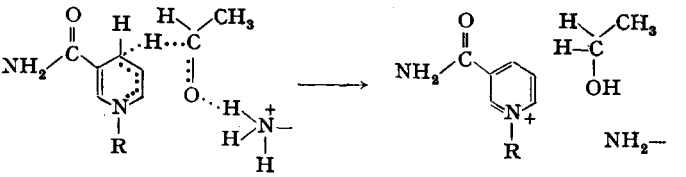
(1)

It is postulated that the shift observed in the DPNH spectrum when complexed is due to the proximity of a positive charge, and it is further proposed that the positive charge resides on the nitrogen of an alkylammonium ion, *e.g.* the ε-amino group of a lysine. A precise location for the alkylammonium ion in the DPNH–enzyme complex is chosen on *independent* grounds. It is reasonable that the alkylammonium ion would form (at least) one hydrogen bond to the DPNH molecule, one to the non-bonding sp^3 electrons of the oxygen in the ring of the ribose to which the dihydropyridine ring is attached. Another hydrogen bond is readily possible to the sp^2 non-bonding oxygen electrons of an acetaldehyde molecule placed so that the carbonyl carbon is directly above the 4-carbon of the dihydropyridine ring, allowing direct hydrogen transfer from DPNH to the aldehyde[1]. From models and scale drawings, the nitrogen of an alkylammonium ion hydrogen bonded to two groups as described would be *ca.* 3.0 Å from the ring nitrogen of the dihydropyridine ring. It can be estimated (the calculations are briefly described below) that a positively charged nitrogen 3.0 Å from the nitrogen of a dihydronicotinamide ring would produce an increase in transition energy amounting to *ca.* 5.2 kcal/mole. The shift observed for a number of DPNH–enzyme complexes is between 3.3 and 3.9 kcal/mole[9]. Although a number of assumptions must be made in order to calculate the expected change in transition energy, it is thought that the agreement is sufficiently good to support the proposition of this section, and therefore to permit the inclusion of an alkyl-ammonium ion as a key component of the "active site" of alcohol dehydrogenase.

The hydrogen-bonded arrangement for the alkylammonium ion along with a binding site for the oxygen of the 3-carbamido group on the enzyme would strongly favor reaction of the acetaldehyde on only one side of the ring (the sides are distinguished through the use of deuterium as a label at the 4-position). It is not necessary, thus, for the dihydropyridine ring to be non-planar[15] and in fact, basicity measurements on 1,4,4-trimethyl-1,4-dihydropyridine suggest sufficient stabilization for planarity in the 1,4-dihydropyridine ring[16].

Stereospecificity with respect to acetaldehyde has been explained as being due to the presence of a "hydrophobic" region (*i.e.*, alkyl chains of certain amino acids) on one side of the "active site". Good evidence in support of such a "hydrophobic region" at the "active site" of alcohol dehydrogenase is found in the discovery of complex formation between long-chain fatty acids amides, DPNH, and enzyme (as well as long-chain fatty acids, DPN, and enzyme)[17].

A simple version of the DPNH-acetaldehyde transition state can be written as in Eqn. 2, and is very much like an equation written on far less sophisticated grounds five years ago[18], and exactly what might have been proposed on the basis of stoichiometry[1]. A simplified representation of the "active site" in the reaction of DPNH and acetaldehyde is shown in Fig. 2.



(2)

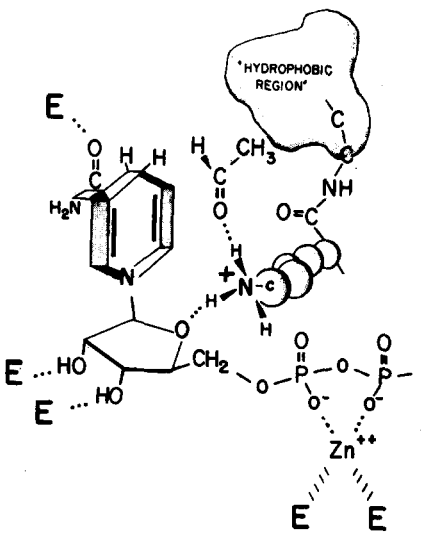


Fig. 2.

Calculations

The quantity which must be estimated is the increase in positive charge of the ring nitrogen of the dihydronicotinamide produced by light absorption. If we take the

mutual potential energy of the solute dipole, μ_1, and the cybotactic region[19] dipole, μ, as $-2\ \mu_1\mu/r^3$ (ref. 20) and *assume* that the same cybotactic region dipole for an alkylpyridinium iodide ion-pair (with solute dipole, μ_2) can be used, the energy required to remove the first solute dipole, μ_1, can be derived by simple proportion, $E_1/E_2 = \mu_1/\mu_2$. Extensive studies on the solvent sensitivity of the charge-transfer transition of 1-alkylpyridinium iodides[19] have led to the estimate that the ion-pair dipole ($\mu_2 = 13.9\ D$) would require 17.3 kcal/mole for removal from the cybotactic region dipole (μ) of water. From the proportion, the energy E_1 for 1-benzyldihydronicotinamide in water, is estimated as 4.9 kcal/mole.

As in previous uses of excited state dipoles[13,14], the mutual potential energy for the instantaneous excited state dipole of the 1-benzyldihydronicotinamide, E_3, is found from the transition energy difference between the least polar solvent (diethyl ether) and the most polar solvent and the energy, E_1 (see Fig. 1). Thus, E_3 is 8.9 kcal/mole. (The excited state dipole measured with the use of fluorescence data is that of the equilibrium excited state[21,22]). The dipole of the instantaneous excited state is found to be 7.2 D by the proportion given above.

The partial moment for the dipole with ring nitrogen positive and carbamido carbonyl oxygen negative was found by taking an angle of 56° with the amide moment (taken as 3.0 D, ref. 6) and finding that dipole which gave a resultant of 3.89 D (ref. 6). The same procedure was followed for the excited state, assuming that the amide partial moment was the same as in the ground state. The resulting partial moments were μ_{ground} 5.2 D and μ_{excited} 9.0 D. From these moments and the equation, $\mu = er$, the change in positive charge at nitrogen, and negative charge at oxygen was estimated as $0.76 \cdot 10^{-10}$ e.s.u. Given an alkylammonium ion at 3.0 Å, and a "dielectric constant", D, of 2, the increase in transition energy of the 1-benzylhydronicotinamide was found to be 8.78 (repulsion) — 3.56 (attraction) or 5.2 kcal/mole. The nitrogen–oxygen distance in the partial dipole of the dihydronicotinamide was taken as 5.0 Å, while the distance of the carbonyl oxygen to the alkylammonium ion was taken as 7.4 Å, with the amide group on the side of the dihydropyridine ring opposite to that where the alkylammonium was located. (The agreement between the calculated and observed ΔE_T is improved if the alternate position for the amide group is chosen).

ACKNOWLEDGEMENT

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METABOLISM OF PHOSPHOLIPIDS

V. STUDIES OF PHOSPHATIDIC ACID PHOSPHATASE*

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SUMMARY

1. The distribution of phosphatidic acid phosphatase is studied in the liver, kidney, brain, and intestinal mucosa of the ox, pig, rabbit, guinea-pig, and rat. Cell fractionation of pig kidney revealed that 63% of the total activity is in the microsomal fraction.

2. The microsomal enzyme from pig kidney is particulate, shows a pH optimum at 6.0, has a K_m of $2.2 \cdot 10^{-4}$ for phosphatidic acid, and is inhibited by fluoride ions. SH groups of the enzyme seem to be essential for activity.

3. Using the microsomal enzyme, the effect of metal ions, chelating agents, detergents, some hydrolytic enzymes, autolysis, and of some higher alcohols is investigated.

4. Procedures for the partial purification and solubilisation of the enzyme are described. The purified enzyme is highly specific for phosphatidic acid and hydrolyses only the phosphoric acid ester bond.

INTRODUCTION

Phosphatidic acid occupies an important position in pathways of phospholipid biosynthesis. It has been shown to be the direct lipid precursor of phosphatidyl inositol[1]

Abbreviations: PA phosphatase, phosphatidic acid phosphatase; TCA, trichloracetic acid.
* Part IV, see ref. 13.