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EFFECTS OF DEUTERIUM ON MALIC DEHYDROGENASE*

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Abstract—Four samples of DPNH containing deuterium in different positions were compared with the protium compound in a bacterial malic dehydrogenase system, tested in both H_2O and D_2O. In H_2O, there was a primary isotope effect on the maximum velocity of about 2 (V_H/V_D) with α-DPND, and a somewhat smaller effect on the Michaelis constant. There was negligible influence on either V_{max} or K_M with β-DPND. In D_2O, however, the effects on K_M were greater than those on V_{max}, and there was also evidence of a stronger secondary isotope effect. The solvent effects of D_2O on V_{max} were nearly the same for all coenzymes, although the effects on the Michaelis constants were more pronounced for those with secondary deuterium substitution.

THE EFFECTS of replacement of hydrogen in biological systems must depend to a large extent upon the kinetic isotope effect;[1] although the effect of deuteration on hydrogen bonding in macromolecules[2] may be of considerable importance in cell division and differentiation,[3] the metabolic effects of deuterium are very probably the result of altered rates of reactions involving hydrogen transfer.

Considerable information has been accumulated on the consequences of replacement of hydrogen by deuterium in enzymatic reactions. Several reports have been concerned with the effects of deuteration of DPNH, for which two monodeuterio derivatives, α-DPND and β-DPND, are known. Because of the stereospecificity of DPN-linked dehydrogenases, the enzymatic oxidation of DPND will remove either all or none of the deuterium from a given stereoisomer;[4] hence for a given dehydrogenase either a primary or a secondary isotope effect can be studied by appropriate choice of DPND. Both Mackler[5] and Shiner *et al.*[6] have observed different effects of α- or β-deuterium substitution in various enzyme systems.

This report is concerned with the effects of several deuterated preparations of DPNH, as well as D_2O, on malic dehydrogenase obtained from a bacterial source.

METHODS

Enzyme assays were carried out at 25 °C, with a Beckman spectrophotometer equipped for automatic recording of absorbance changes at 340 mμ. The assay system consisted of 0·016 M tris buffer (pH 7·4), 0·003 M $MgCl_2$, 0·001 M oxalacetate, 50 to 100 μg enzyme, and varying concentrations of DPNH. The enzyme, a gift from Dr. Robert W. Swick, was a preparation from *Propionibacterium shermanii*. The preparation contained negligible alcohol or lactic dehydrogenase or DPNH oxidase activities.

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Initial rates were obtained by extrapolation to zero time, and the values for the maximum velocities and the Michaelis constants were obtained by the Lineweaver–Burk method of plotting the reciprocal of the velocity against the reciprocal of the concentration. The values were calculated by the method of least squares.

From two to ten experiments were carried out with each sample, each experiment employing five to seven different concentrations of coenzyme. The data from individual experiments were pooled for calculation of the K_M and V_{max} values. We feel that the values thus obtained are more meaningful than averages from individual experiments, since an aberrant datum would have less weight in the former procedure.

Five samples of DPNH were used in these experiments. These included: (1) DPNH, a commercial sample (Sigma); (2) α-DPND, prepared by reduction of DPN with CD_3CD_2OD in the presence of alcohol dehydrogenase; (3) β-DPND, prepared by enzymatic reduction of DPN-4-d with CH_3CH_2OH; (4) $DPND_2$, prepared by enzymatic reduction of DPN-4-d with CD_3CD_2OD so that both hydrogen atoms in the 4-position were replaced by deuterium; and (5) α,β-DPND, prepared from DPN by hydrosulfite reduction in D_2O. The deuterated DPN (DPN-4-d) was obtained by formation of the cyanide-addition compound in D_2O. In this compound, the hydrogen on the 4-carbon of the pyridine ring is labile, and readily exchanges with deuterium, so that when the cyanide compound is then decomposed, the regenerated DPN contains an atom of deuterium in this position.[7] The deuterium content of the various DPND samples was not measured. On the basis of ultraviolet absorption at 260 and 340 mμ, the purity of the various preparations was established. The α-DPND and α,β-DPND samples were about 98 per cent pure; the β-DPND and $DPND_2$ were about 85 per cent pure, the principal contaminant being adenylic acid. A sample of DPNH, prepared by enzymatic reduction of DPN previously incubated in alkaline cyanide (in H_2O), also about 85 per cent pure, did not differ appreciably from the commercial sample (97 per cent pure) when assayed in this system at the same concentration (measured by absorption at 340 mμ).

All coenzyme and substrate solutions were freshly prepared and standardized immediately before use. Fresh dilutions of the enzyme from a stock solution also were used in each experiment.

When D_2O was used as the solvent, the buffer was prepared by lyophilizing the tris buffer used in H_2O, then resuspending in D_2O. The solution in D_2O gave a reading of pH 7·6, which is equivalent to pD 8·0.[8] The pH and pD values employed are those of apparent maximum enzyme activity.

The reaction mechanism of the enzyme used here is not known. However, for a variety of mechanisms, the kinetic equations reduce to the following form when the concentration of one substrate (i.e. oxalacetate) is at a saturation level:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \times \frac{1}{C}$$

where v is the initial reaction velocity observed at a given concentration of the other substrate (in this case the coenzyme) C, V_{max} is the maximum velocity, and K_M is a constant, the significance of which depends on the reaction mechanism. For the mechanism in which a rapid equilibrium is established among the enzyme, coenzyme,

and substrate, K_M is the dissociation constant for the reaction ES + C $\rightleftarrows$ ESC; whereas for various steady-state mechanisms K_M is a function of a number of kinetic constants.

Since the preparation designated α,β-DPND represents an equimolar mixture of the α- and β-stereoisomers, the velocity equation can be written:

$$v = \frac{V_\alpha C_\alpha}{C_\alpha + K_\alpha (1 + C_\beta/K_\beta)} + \frac{V_\beta C_\beta}{C_\beta + K_\beta (1 + C_\alpha/K_\alpha)}$$

where the subscripts refer to the individual values for α- and β-DPND. The same form of equation is obtained for either a steady-state[9] or an equilibrium[10] mechanism.

When $C_\alpha = C_\beta = 0{\cdot}5\ C_t$ (the total substrate concentration) the Lineweaver–Burk transformation becomes:

$$\frac{1}{v} = \left(\frac{K_\alpha + K_\beta}{K_\alpha V_\beta + K_\beta V_\alpha}\right) + \left(\frac{2K_\alpha K_\beta}{K_\alpha V_\beta + K_\beta V_\alpha}\right) \times \frac{1}{C_t}$$

Therefore, for the α,β-mixture, the apparent V_{max} is equal to $(K_\alpha V_\beta + K_\beta V_\alpha)/(K_\alpha + K_\beta)$; and the apparent K_M is equal to $2K_\alpha K_\beta/(K_\alpha + K_\beta)$. Thus the data obtained from the individual stereoisomers can be used to calculate the K_M and V_{max} values for the mixture.

The isotope effect in terms of Michaelis constants and maximum velocities is given by:

$$\frac{v_H}{v_D} = \frac{V_H C_H/(C_H + K_H)}{V_D C_D/(C_D + K_D)}$$

When the substrate concentrations are equal, and large in respect to K_D or K_H, the expression simplifies to $v_H/v_D = V_H/V_D$. However, when C is small in respect to K, the limiting expression is then:

$$\frac{v_H}{v_D} = \frac{V_H}{V_D} \times \frac{K_D}{K_H}$$

so that a larger effect on K_H/K_D than on V_H/V_D would indicate an inverse isotope effect at low values of C. These relationships were first pointed out by Salzer and Bonhoeffer.[11]

RESULTS

Table 1 shows the Michaelis constants and maximum velocities for the five samples of DPNH in H_2O and in D_2O. In H_2O there was a maximum isotope effect (V_H/V_D) of a little less than 2 with α-DPND, but with β-DPND there was, if anything, a slight inverse effect. These results indicate that this enzyme is α-stereospecific. The data obtained with the doubly deuterated sample, $DPND_2$, did not differ appreciably from those for α-DPND, an indication that β-substitution of deuterium had little effect on the reaction. For all four deuterated samples there was less effect on the Michaelis constant than on the maximum velocity, so that a normal isotope effect would be expected at all coenzyme concentrations.

TABLE 1. KINETIC AND SOLVENT EFFECTS OF DEUTERIUM ON MALIC DEHYDROGENASE*

Coenzyme	Isotope effects in H_2O					Isotope effects in D_2O					Solvent effects	
	No.	K_M	V_{max}	K_H/K_D	V_H/V_D	No.	K_M	V_{max}	K_H/K_D	V_H/V_D	K_{H_2O}/K_{D_2O}	V_{H_2O}/V_{D_2O}
DPNH	62	1·73 ± 0·049	28·7 ± 0·44	—	—	32	2·23 ± 0·110	18·9 ± 0·53	—	—	0·74	1·51
α-DPND	35	1·02 ± 0·049	15·5 ± 0·23	1·70	1·85	14	1·17 ± 0·138	10·2 ± 0·38	1·90	1·52	0·86	1·52
β-DPND	34	2·11 ± 0·089	29·6 ± 0·73	0·82	0·97	14	1·48 ± 0·166	17·3 ± 0·77	1·51	1·09	1·42	1·71
α,β-DPND Observed	21	1·37 ± 0·033	20·4 ± 0·29	1·27	1·41	25	1·43 ± 0·054	13·0 ± 0·20	1·55	1·45	0·95	1·57
Predicted		1·37 ± 0·048	20·1 ± 0·58	1·26	1·43		1·31 ± 0·108	13·3 ± 0·52	1·71	1·42	1·05	1·51
$DPND_2$	27	1·14 ± 0·058	14·6 ± 0·26	1·51	1·97	22	0·63 ± 0·113	9·0 ± 0·29	3·56	2·10	1·82	1·62

* Values for K_M are in moles ($\times 10^5$) with standard errors; those for V_{max} are μmoles/min/mg N.
"No." refers to the total number of experimental points; i.e. individual concentrations of coenzyme, not the number of experiments.

However, in D_2O the situation was considerably different, in that the effects on K_M were greater than those on V_{max}. This observation means that at low coenzyme concentrations there would be inverse isotope effects in D_2O.

It is also of interest to note that the influence of β-substitution of deuterium is more important in D_2O than in H_2O, since in the former solvent there were significant differences between the K_M values for DPNH and β-DPND on the one hand, and between those for α-DPND and $DPND_2$ on the other; i.e. there is a definite secondary isotope effect in D_2O that is not seen in H_2O.

The solvent effects of D_2O on maximum velocity were nearly the same for all coenzymes. The effects on the Michaelis constants were pronounced for β-DPND and $DPND_2$ but rather slight (and in the opposite direction) for α-DPND, α,β-DPND, and DPNH. It should be remarked that this procedure of measuring solvent effects at the pH or pD of maximum activity has been criticized by Srere *et al.*,[12] who prefer to make comparisons at the same pH or pD. Their reason is that pD "has a firm and simple significance as the activity of the cationic form of the hydrogen isotope. . . ." However, pOD should have similar significance; and when pH equals pD, pOH does *not* equal pOD, since the dissociation constants of D_2O and H_2O differ by a factor of 5.

The results obtained with α,β-DPND in both H_2O and D_2O agree well with the values calculated from the data on the individual stereoisomers. This agreement provides a test of internal consistency of the results and also indicates that the amount of DPNH in these samples must have been negligible.

DISCUSSION

These experiments do not give a complete picture of the effect of deuterium on reactions requiring DPNH, since only one concentration of substrate (oxalacetate) was used. Furthermore, since the mechanism of action of this enzyme is not known, it is impossible to state whether K_M represents the dissociation constant for the reaction $ES + C \rightleftarrows ESC$ or whether it is a function of several kinetic constants. If the reaction obeys an equilibrium mechanism rather than a steady-state mechanism, then the effect of deuterium ($K_{DPNH}/K_{\alpha\text{-DPND}}$) is very similar to that reported by Shiner *et al.*[6] for yeast alcohol dehydrogenase. For this enzyme the replacement of DPNH by α-DPND resulted in a ratio of maximum velocities (V_H/V_D) of 1·75, and a ratio of K_H/K_D of 1·34, where the K's are the equilibrium constants for the reaction shown above. Thus, although the data reported here do not permit any decision to be made concerning the reaction mechanism, there is sufficient information to predict the direction and magnitude of the isotope effect under various conditions.

The effects of deuteration of DPNH on malic dehydrogenase are qualitatively similar to those seen not only with alcohol dehydrogenase but also with lactic dehydrogenase;[13] it seems probable that the results obtained on these three enzymes are characteristic of DPNH-linked enzymes as a group.

In none of these three cases does the primary isotope effect (V_H/V_D) approach the theoretical maximum value of 6 predicted by Wiberg.[1] The fact that the values are only about one-fourth to one-third of this maximum indicates that there is considerable conservation of zero-point energy in the transition states for these reactions.[6]

The solvent effect with this enzyme is considerably less than that reported for malic dehydrogenase by Srere *et al.*,[12] who observed that at pH 7·5 the rate of reduction of

oxalacetate in D_2O was decreased to about 20 per cent of the normal rate. It is probable (although these authors did not specify) that their enzyme was obtained from a mammalian rather than a bacterial source and hence might be expected to respond differently in D_2O.

The prediction of effects of metabolic inhibitors *in vivo* from observations *in vitro* is at best a hazardous occupation. For the deuterated organism the situation is even more complex: not only are there both solvent and substrate effects to contend with, but also effects arising from deuteration of the enzyme that may alter the effects of substrate deuteration.[14] In partially deuterated systems there will be mechanisms for discriminating between protiated and deuterated substrates, generally favoring the accumulation of the latter. Spirtes and Eichel[15] have reported that the DPNH content of rat liver is 345 μg/g fresh weight; i.e. about 0·0005 M. This amount, of course, is distributed among a large number of enzymes. The total concentration of enzymes acting on DPNH is probably considerably below the concentration of the coenzyme; however, the problems of cytoplasmic localization of enzymes and coenzymes are such that it is difficult to predict results *in vivo* from measurements made *in vitro*. Furthermore, the relative amounts of α- and β-DPNH-specific enzymes are not known.

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