Tetrahedron Vol. 42, No. 4, pp. 993 to 1006, 1986
Printed in Great Britain.

0040-4020/86 $3.00 + .00
Pergamon Press Ltd.

DEHYDROGENATION OF FORMATE BY 10-METHYLACRIDINIUM ION[1a]

by

Jonathan E.C. Hutchins*[1b], David A. Binder, and Maurice M. Kreevoy*[1c]

Contribution from the Chemical Dynamics Laboratory of
the Department of Chemistry
University of Minnesota
207 Pleasant St. S.E.
Minneapolis, Minnesota 55455
U.S.A.

(*Received in USA* 4 *July* 1985)

ABSTRACT

Formate is dehydrogenated to CO_2 by 10-methylacridinium ion, mimicking formate dehydrogenase. The hydrogen and carbon kinetic isotope effects are 2.74 and 1.027 in a mixed solvent consisting of dimethylformamide and water in a 4:1 ratio, at 50°. These values are similar to those observed in the enzymatic reaction (2.27 and 1.042, respectively) suggesting that the mechanisms of enzymatic and nonenzymatic reactions are the same, and transition state structures not too different. Marcus theory of atom and group transfer is used to locate the transition state for the nonenzymatic reaction 0.4 of the distance along the minimum energy path from precursor configuration to successor configuration. It is concluded, following Cleland and coworkers, that the protein of the enzyme dehydrates the formate and deforms the cofactor NAD^+ so as to make the reaction more spontaneous. This produces an enzymatic transition state in which the covalency changes around hydrogen are less advanced than in the non-enzymatic transition state, but the environment of the carboxylate is much more suitable to the product, CO_2.

Yeast formate dehydrogenase brings about the oxidation of formate to CO_2, in the process, transferring a hydride ion to the enzyme cofactor, Nicotinamide Adenine Dinucleotide (NAD^+), reducing the latter to the corresponding 1,4-dihydropyridine, NADH (eq. 1)[2]

$$HCO_2^- + NAD^+ \rightarrow CO_2 + NADH \qquad (1)$$

Cleland and coworkers have made extensive studies of isotope effects on these reactions.[3,4] They have shown that the reaction is essentially irreversible, and that the step involving the rearrangement of covalent bonds is fully rate-limiting.[4] They have found that the NAD^+ could be replaced with other pyridinium ions, and measured the changes in isotope effects which attended these replacements.[3] They have found that N_3^- is a much more effective competitive inhibitor than NO_3^-.[4] From these studies they have reached the following conclusions:[3]

1.) Prior to the hydride transfer, the pyridinium ring is strongly distorted in the direction of dihydropyridine geometry, considerably increasing its hydride affinity (reduction potential).

2.) The transition state in the enzyme catalyzed reaction resembles the products much more closely than the reactants.

3.) Relatively small increases in the reduction potential of the cofactor significantly shift the transition state structure toward that of the reactants.

Attempts to oxidize formate with simple NAD^+ analogues have been unsuccessful[5] but the reduction potential of 10-methylacridinium ion is ~240 mv larger than that of NAD^+[6] which should facilitate the reaction, according to Cleland's point 1.). Further, in refluxing formic acid solvent, formate has been shown to reduce 10-methylacridinium ion.[7] We now report that formate is oxidized by 10-methylacridinium ion at measurable rates in both isopropanol (IPA) - water (4:1 by volume) and dimethylformamide (DFM) - water (4:1 by volume) at 50° or 25°C. $^2HCOO^-$ and $H^{13}COO^-$ isotope effects have been measured and compared with those observed in enzymatic reactions. These isotope effects suggest that the nonenzymatic reactions are similar to the enzymatic reaction in general mechanism but different in details of transition state (TS) structure.

In earlier work the Marcus theory of atom and group transfer[8-10] has been shown to give a reasonably faithful account of the rate constants for hydride transfer between NAD^+ analogues,[11-14] including isotope effects.[12] This theory is capable of giving semiquantitative insight into TS structure.[13] In the present paper we apply this theory to the oxidation of formate by NAD^+ and its analogues. We conclude that

Cleland's first point, the exaltation of the hydride affinity of the cofactor, is strongly supported. We suggest that the second point, on the location of the TS on the minimum energy path, is valid only with respect to the structure and solvation of the CO_2 unit __ not with respect to the making and breaking of C-H bonds. Marcus theory indicates much less responsiveness of TS structure to hydride affinity than Cleland's third point would imply. The isotope effects observed with the unnatural cofactors were closer to our present results, with a nonenzymatic system, than were those obtained in the natural system. We therefore suggest that the changes observed on substitution of the unnatural cofactors into the enzymatic system are principally the result of nonideal protein-cofactor binding, rather than a response to the increased hydride affinity of the unnatural cofactors.

EXPERIMENTAL

Materials and Solutions

Formic acid solutions were prepared from reagent grade 90% formic acid (Spectrum Chemical Mfg. Co.) analyzed by titration against standard NaOH (found: 90.4% w/w acid). Formic acid-d_1 (M.S.D. Isotopes, 99% atom D) titrated similarly as 96.2% w/w acid. Proton NMR was used to check the H content of the latter material by comparing OH/CH peak integral ratios (50 transients) of 12% w/w solutions of DCOOH and HCOOH in DMSO-d_6 containing 1.3% w/w H_2O. The formic acid-d_1 was found to have 0.91 mole% H. Standard NaOH solutions were made from "Dilut-it" analytical concentrate (J.T. Baker Chemical Co.) and boiled-out, redistilled water. Aqueous formate buffers having 5 times the desired final concentrations were made from the reagents described above. One volume of aqueous buffer was then diluted with four volumes of either redistilled [12] spectrograde IPA or DMF (MCB "OmniSolv"), generally immediately before use. Sodium perchlorate was also added, as needed, to maintain an ionic strength, μ, of 0.13M. 10-Methylacridinium iodide was prepared by methylation of acridine,[11] and had appropriate spectroscopic characteristics.

pH Measurement

Buffer pHs and pHs of reacted solutions were determined at 50° or 25° using a standardized Radiometer TTTlc pH meter and GK 2322C combina-

tion electrode. Measured pHs at 50° in 4:1 v/v DMF-H_2O were corrected by subtracting 0.69. The corresponding correction for 4:1 v/v IPA-H_2O was -0.10. These corrections were determined by measuring pHs of 10^{-2} and 10^{-3} M $HClO_4$ in these solvents at 50°, with ionic strength also adjusted to 0.13 using $NaClO_4$.

Kinetic Method

10-Methylacridinium iodide was delivered as 12μL of a stock solution (1-2 x 10^{-2} M in spectrograde CH_3CN) to 3mL of appropriate buffer at 50° ± 0.1° or 25° ± 0.1° in quartz spectrophotometer cells. The cells were then tightly stoppered and the reaction observed in the thermostatted cell compartment of either a Beckman DU/Gilford or a Perkin Elmer λ3B spectrophotometer. Loss of acridinium ion was monitored at 358 nm: appearance of 10-methylacridan was monitored at 284 nm. Kinetic experiments were generally followed to at least 95% reaction. In the presence of a large excess of formate, these absorbance changes were accurately described by the pseudo first-order rate law.[15] Data were analyzed by an iterative least squares curve-fitting program which searches for that infinite-time absorbance which minimizes the sum of the squares of the residuals between calculated and experimental absorbances. Residuals of ± 0.002 absorbance units or less were so obtained. CO_2 evolution was also monitored in a few cases at 50° in both 4:1 v/v DMF-H_2O and 4:1 v/v IPA-H_2O, using a differential gas manometer.[16]

Product Analysis

The electronic spectra of product solutions were obtained using either a Cary 219 or Perkin Elmer λ3B spectrophotometer. Quantitative matches were obtained between these spectra and spectra of solutions of 10-methylacridan of the appropriate concentrations in the same buffer.

pK_R of 10-Methylacridinium Ion

The pK_R of this ion in 4:1, DMF:H_2O, at 50° was determined from the absorbance at 358 nm of six 10-methylacridinium iodide solutions, 5.30 x 10^{-5} M, each buffered to a pH between 4.7 and 8.1 with an acetic acid-acetate buffer.[13] Since, at the higher pH's, the absorbances drifted toward lower values with time, values obtained immediately after mixing were used. The absorbance was quite unstable at still higher pH, so the limiting absorbance of 9-hydroxy-10-methyl-acridan could not be obtained directly. The value was obtained by a trial-and-error procedure; a value was selected; an average value of K_R was obtained and the absorbance was calculated at each of the 6 pH's. The final value of the limiting absorbance was chosen so as to minimize the discrepancies be-

tween calculated and observed absorbances. The best average value of pK_R was 6.7.

Apparent pK_{for}

The apparent dissociation constants, K_{for}, of formic acid in the DMF-H_2O mixture and the IPA-H_2O mixture, both at 50° and an ionic strength of 0.13 M, were determined from the pH of 1:1 buffers.[17] In the DMF-H_2O mixture pK_{for} was 5.84 and in IPA-H_2O it was 4.91.

The Hydrogen Isotope Effect

The primary hydrogen isotope effect was obtained from rate constants determined under identical conditions with formate and formate-d_1. These experiments used half neutralized formate solutions. Experiments with formate and formate-d_1 were carried out side by side, in the spectrophotometer, at the same time, and isotope effects were evaluated by taking the ratios of rate constants so determined, so as to minimize the possible effect of imperfectly controlled temperature.

The Carbon Isotope Effect

The $^{12}C:^{13}C$ isotope effect was determined by isolating the product CO_2 from reactions in which only a small fraction, f, of the formate was oxidized. Another portion of the same sample of formate was completely oxidized by an excess of I_2 in DMSO[3]. The $^{13}C:^{12}C$ ratios of both CO_2 samples, R_t amd R_∞, respectively, were determined mass spectrometrically using a Finnegan MAT Delta E isotope ratio mass spectrometer.[18,19] The reference was a standard in which $^{13}C:^{12}C$ is 0.01124.[20] (R_∞ was 0.010882.) The ratio of rate constants, k_{12}/k_{13}, was given by eq. 2.[18]

$$\frac{k_{12}}{k_{13}} = \frac{\log (1-f)}{\log (1-fR_t/R_\infty)} \qquad (2)$$

The reactions were carried out on a scale of ~200 cm^3. The solutions must be completely purged of CO_2 before reactions, and, after reaction, all the product CO_2 must be collected to avoid isotopic fractionation of CO_2 in its distribution between the solution and the vapor phase. Two methods were used for these CO_2 transfers, both described in detail by O'Leary.[18] In one, 3 or 4 freeze (with liquid N_2), pump, collect, thaw cycles were used. In the other method, solutions were sparged with high purity N_2 for two hours. In both methods the CO_2 for analysis was collected in a liquid N_2 cooled cold trap. In the second method the volatilization of the CO_2 after the reaction was encouraged by the addi-

tion of enough 4.5 M H_2SO_4 to lower the pH to 2. This was introduced through a stopcock port protected by a septum cap. The CO_2 samples were freed of solvent and other extraneous vapors by distillation between IPA-dry ice and liquid N_2 cold traps before introduction into the mass spectrometer.

RESULTS

Products

In both IPA-H_2O and DMF-H_2O, at either 25° or 50°, 10^{-3} to 10^{-5} M 10-methylacridinium ion is, over a period of time, quantitatively converted to 10-methylacridan by an excess of formate ion. The electronic spectra of reactants and products were quantitatively identical with those of authentic materials. CO_2, identified by its mass spectrum, is the other product. Rates measured by spectrophotometrically monitoring the disappearance of 10-methylacridinium ion, or the appearance of 10-methylacridan, or gasometrically monitoring the appearance of CO_2, were the same within their experimental uncertainties. Over the periods required for 7-10 half lives of the reactions with formate, the spectrum of 10-methylacridinium ion in the absence of formate, but otherwise under the same conditions (solvent, temperature, pH) was essentially unchanged. We therefore conclude that eq. 3 adequately and completely describes the transformations being observed. (Ac^+ is the 10-methylacridinium ion; AcH is 10-methylacridan.)

$$Ac^+ + HCO_2^- \rightarrow AcH + CO_2 \qquad (3)$$

This reaction has been previously reported under other conditions.[7]

Rates in DMF-Water

Pseudo first-order rate constants, k_1, measured in 4:1, DMF:H_2O, v:v at 50°, with 1:1, formic acid:formate buffers, were directly proportional to the apparent formate concentration, F_{for}, as shown in fig. 1.

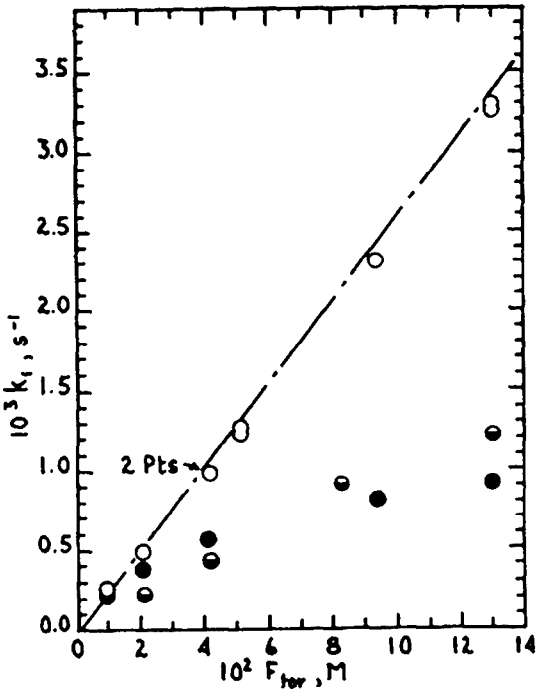

Figure 1. Pseudo first-order rate constants in 4:1, DMF:H_2O, at 50°, as a function of F_{for}. Open circles are for 1:1, formic acid: formate buffers; half filled are for 1:10 buffers; filled are for 10:1 buffers. The dot-dashed line is the locus of 1:1 buffer points plotted against C_{for}, instead of F_{for}, and shows the smallness of the corrections for formate complexing except for 10:1 buffers.

These solutions all had pH values between 5.81 and 5.88, averaging 5.84. The proportionality constant, k_2'' is 2.4 x 10^{-2} $M^{-1}s^{-1}$. With 1:10., formic acid:formate buffers the average pH was 7.02, with no solution deviating by more than .02 units, and analogous results were obtained, but k_2'' was 1.01 x 10^{-2} $M^{-1}s^{-1}$. Moreover, with 10:1, formic acid: formate buffers the pH decreased steadily from 4.8 to 4.2 with increasing buffer concentration, and k_1 was as strongly nonlinear function of F_{for}. These results are given in Table I and fig. 1.

TABLE I. Rate as a Function of Formate Concentration with a 10-Fold Excess of Formic Acid[a]

$10^2 F_{for}$, M	pH	$10^4 k_1$,[b] s^{-1}	$10^2 C_{for}$, M[c]	$10^2 k_2'$, $M^{-1}s^{-1}$
1.04	4.71	2.20	0.92	2.39
1.04	4.81	2.57[d]	0.92	2.79
2.08	4.72	3.77	1.60	2.36
4.16	4.60	5.72	2.38	2.40
9.36	4.33	8.16	2.78	2.94
13.0	4.19	9.08	2.62	3.47

(a) In 4:1, DMF:H_2O, v:v, at 50°C.
(b) Determined by monitoring the decay of 10-methylacridinium concentration at 358 nm except where otherwise noted.
(c) Calculated using the measured pH values and the $\underline{K}_1$ and $\underline{K}_2$ values given in the text.
(d) Determined by monitoring the growth of 10-methylacridan concentration at 284 nm.

These results could be rationalized, and a constant second-order rate constant, $\underline{k}_2$, obtained, by taking into account equilibria which are well documented in other solvents. 10-Methylacridinium ion is reversibly hydroxylated in the 9-position, according to eq. 4.[21] (AcOH is 9-hydroxy-10-methylacridan.) And formate ion forms "homoconjugate" complexes with formic acid according to eqs. 5 and 6.[22]

$$Ac^+ + H_2O \overset{K_R}{\rightleftharpoons} AcOH + H^+ \qquad (4)$$

$$HCO_2^- + HCO_2H \overset{K_1}{\rightleftharpoons} HCO_2 \cdot HO_2CH^- \qquad (5)$$

$$HCO_2^- + 2HCO_2H \overset{K_2}{\rightleftharpoons} HCO_2(HO_2CH)_2^- \qquad (6)$$

$\underline{K}_R$ was determined by a standard spectrophotometric method,[6,13] briefly described in the experimental section. $p\underline{K}_R$ has a value of 6.7 in the DMF-H_2O mixture, with a constant ionic strength of 0.13, and a value of 7.74 has been reported for dilute solutions in the IPA-H_2O mixture at 25°.[6] The $\underline{K}_1$ and $\underline{K}_2$ values were obtained from the pH values given in Table I. An iterative protocol, designed to find the most nearly constant, self-consistent values of $\underline{K}_1$ and $\underline{K}_2$ was used. The best values of $\underline{K}_1$ and $\underline{K}_2$ are 1.05 and 2.2, respectively, but these values are heavily dependent on one another, and on the assumption that activity coefficients do not vary with ionic composition, or due to the addition of up to 1.3 $\underline{M}$ acetic acid, as long as the ionic strength is constant. Their individual values are very uncertain, and we cannot even completely exclude the possibility that $\underline{K}_2$ is zero. However they reproduce the measured pH values with an average discrepancy of <.01 when used along with $\underline{K}_{for}$ (5.84). We therefore believe that they also give reasonably accurate values of the molecular formate concentration, $\underline{C}_{for}$, which could be calculated from them, $\underline{F}_{for}$, and the buffer ratio. Table I gives the $\underline{C}_{for}$ and the $\underline{k}_2'$ values, $\underline{k}_1/\underline{C}_{for}$. The average value of $\underline{k}_2'$ is $2.7 \pm .3 \times 10^{-2}\ \underline{M}^{-1}\underline{s}^{-1}$. Since no more than about 1% of the 10-methylacridinium ion would be hydroxylated at these pH's, this $\underline{k}_2'$ is also $\underline{k}_2$, the fully corrected second order rate constant for the reaction of formate ion with 10-methylacridinium ion at 50° in the DMF-H_2O mixed solvent. For the 1:1 buffer the corrections for the complexing of formate are much smaller. The average value of $\underline{k}_2'$ is $2.6 \pm .2 \times 10^{-2}\ \underline{M}^{-1}\underline{s}^{-1}$,

compared to 2.4×10^{-2} for k_2''. Although C_{for} is not a linear function of F_{for}, in this case the differences between the two were not large enough to make k_1 a visibly nonlinear function of F_{for}. At a pH of 5.84, .879 of the nominal 10-methylacridinium ion is in the unhydroxylated form. Multiplying k_2' by 1.14 gives $3.0 \pm .2 \times 10^{-2}$ M^{-1} s^{-1} for k_2. With the 1:10 buffers, no significant fraction of the formate is complexed, so that k_2'' is also k_2'. But at a pH of 7.02 the molecular 10-methylacridinium concentration is only .324 of its formal concentration. When this is taken into account a value of $3.1 \pm .1 \times 10^{-2}$ $M^{-1}s^{-1}$ is obtained for k_2. In all cases the cited uncertainties are the average deviations from the mean values. The precision of the mean values would be better, by factors of 2-3, than the indicated values. However their real accuracy is also encumbered by systematic errors due to inaccuracies in the various equilibrium constants, and the assumption about activity coefficients, so that we believe the agreement among the k_2 values derived at the three different buffer ratios is actually quite satisfactory. The best value of k_2 in the DMF-H_2O mixture at 50° appears to be 2.9×10^{-2} $M^{-1}s^{-1}$, and it appears to be uncertain by ~10%.

Rates in IPA-Water

In the IPA-H_2O mixtures at 50°, 1:10, 1:1, and 10:1, formic acid: formate buffers were again studied. Their solutions gave average pH values of 6.14, 4.96, and 4.02, respectively. Rates were much slower, but k_1 values were again proportional to F_{for} at each buffer ratio up to F_{for} values of 5×10^{-2}. There is no evidence that the formic acid-formate complexing reactions (eqs. 5 and 6) are significant at low concentrations in this solvent, so F_{for} was equated to C_{for}. At 25° K_R for 10-methylacridinium ion is 7.74,[6] so very little of the ion is hydroxylated in any of these solutions. Thus k_1/F_{for} could be approximately equated to k_2. Evaluated in this way k_2 has an average value of $3.4 \pm .4 \times 10^{-4}$ $M^{-1}s^{-1}$. The scatter is somewhat larger for these k_2 values than for those measured in DMF-H_2O mixtures because the reactions were inconveniently slow, and many of the k_1 values were obtained by monitoring less than 10% of the complete conversion.

At high F_{for} values, in IPA-H_2O, systematically and sharply higher values of k_1/F_{for} were observed. We suspect that this is caused by reaction in some sort of ion aggregate, but we are unable to suggest anything attractive.

The Hydrogen Isotope Effect

In the DMF-H_2O mixed solvent, five measurements of the kinetic hy-

drogen isotope effect (substitution at carbon in formate) at 50° were made. All were made with 1:1 buffers; two at $\underline{F}_{for}$ values of 0.130 and three at $\underline{F}_{for}$ of 0.052. An average $\underline{k}_H/\underline{k}_D$ of 2.74 was obtained, with an average deviation from the mean of 0.05 and a probable error of the mean of 0.02. In the same solvent, a single determination of $\underline{k}_H/\underline{k}_D$ at 25° gave a value of 3.14. A single determination of $\underline{k}_H/\underline{k}_D$ at 50° in the IPA-H_2O solvent gave 2.78. None of these values is corrected for the small percentage (~1%) of $HCOO^-$ in our $DCOO^-$, so, in all cases, the true values may be assumed to be ~3% higher than those reported.

The Carbon Isotope Effect

The effect of isotopic substitution of the formate carbon was determined in five experiments in the DMF-H_2O solvent; two at 50° and three at 25°. Buffer ratios were 1.0, $\underline{F}_{for}$ was 8-52 x 10^{-3} and methylacridinium iodide concentrations were 6-20 x 10^{-4}. The average value of $\underline{k}_{12}/\underline{k}_{13}$ was 1.0267 with an average deviation from the mean of 0.0010 and a probable error of 0.0005. There was no evident ordering of results according to temperature or concentration.

DISCUSSION

The isotope effects on the dehydrogenation of formate by 10-methylacridinium ion in DMF:H_2O, 4:1, are compared, in Table II, with the

Table II. Isotope Effects on Formate Dehydrogenation

Oxidant	$\underline{k}_H/\underline{k}_D$	$\underline{k}_{12}/\underline{k}_{13}$
10-methylacridinium ion	2.74[b]	1.027[c]
NAD^+	2.27	1.042
deamino-NAD^+	2.40	1.042
thio-NAD^+	2.36	1.038
pyridinealdehyde-NAD^+	2.86	1.041
acetylpyridine-NAD^+	3.12	1.036

(a) Taken from ref. 4, and measured in water at 25°, except where otherwise stated.
(b) Present work, measured in 4:1, DMF:H_2O, at 50°.
(c) Present work, measured in 4:1, DMF:H_2O, at 25° and 50°.

isotope effects on the enzymatic reactions of NAD^+ and its analogues. They are similar enough to strongly support the hypothesis that the enzymatic and nonenzymatic reactions are generally similar, although some difference in TS structure is suggested. Recent work has shown that hydride transfer to and from NAD^+ and its analogues is a one step reaction, not involving radicals or radical ions as intermediates unless the

other reactant is strongly predisposed toward one-electron reactions.[11,14,23-25] This is not the case for formate, so we conclude that both enzymatic and nonenzymatic formate dehydrogenation are one-step hydride transfers.

We conclude that the protein of formate dehydrogenase does substantially increaase the spontaneity of the rate limiting step, as suggested by Cleland and coworkers.[3] 10-Methylacridinium ion is a more powerful hydride acceptor (2-electron oxidizing agent) than NAD^+ by a factor of $\sim 10^8$ (240 mv).[6] When it is substituted for NAD^+ the reaction occurs without the protein. As noted, the isotope effects suggest considerable similarity between the TSs of the enzymatic and nonenzymatic reactions.

It seems quite unlikely that the nonenzymatic reaction has a "late" TS. Using a reduction potential of -78 mv for 10-methylacridinium ion[6] and -420 mv for CO_2(aq)[26] an equilibrium constant, $\underline{K}$, of 10^{12} is calculated for this reaction. Both reduction potentials are for aqueous solution at pH of 7, and the reaction shown in eq. 3 does not involve H^+, so pH is not a problem, however several other problems combine to make $\underline{K}$ uncertain (probably too small) by several powers of 10. Equation 3 represents a combination of ions, which is certain to be more spontaneous in our partially aqueous solution than in pure water. The use of DMF, in particular, probably destabilizes formate relative to its reaction products. Finally, although the value cited for the reduction potential of CO_2(aq.) seems to be accepted,[3] it is not entirely clear what standard state is used for CO_2(aq.).[26] Nevertheless, we proceed with a value of 10^{12} for $\underline{K}$, because only a very rough value is required for the following, semiquantitative discussion. The Marcus parameter, which gives the fractional progress along the minimum energy path, from precursor complex to successor complex, at which the TS occurs, is $\underline{\chi}$, defined in eq. 7.[13]

$$\chi = 0.5\,[1 - (RT/\lambda)\ln K] \qquad (7)$$

$\underline{\lambda}$ is four times the free-energy of activation for a similar but energetically symmetrical reaction.[11] It is not known, in the present case, but hydride transfer between NAD^+ analogues, which is an inherently faster reaction, gives values between 290 and 340 kJ $mole^{-1}$.[11,27] We therefore assume 400 kJ $mole^{-1}$, as a reasonable value for the present reaction. With these estimates a value of 0.4 is obtained for $\underline{\chi}$. The reaction is even slower in pure water than in the IPA-H_2O mixture, and it is presumably even more spontaneous in the mixed solvents than in water, thus 0.4 can be regarded as an upper limit on $\underline{\chi}$. This is at least rough-

ly consistant with k_H/k_D, since this has a value of ~5.4 for nearly symmetrical hydride transfers[12] and is expected to be lower for more spontaneous reactions.[12,14]

It is somewhat harder to interpret k_{12}/k_{13}. The ratio of imaginary frequencies in eq. 8[28] must be very close to 1.000, because H is much lighter than either ^{12}C or ^{13}C.[28] It is also very similar to the ratio of real C-H stretching frequencies, ν_{12}/ν_{13}, so that any non-unit value it might have would largely cancel.

$$\frac{k_{12}}{k_{13}} = P_s \times \frac{\nu^*_{12}\nu_{13}\sin h\,(h\nu_{12}/2kT)}{\nu^*_{13}\nu_{12}\sin h\,(h\nu_{13}/2kT)} \qquad (8)$$

P_s, the product of symmetry factors is exactly unity, so k_{12}/k_{13} would reduce to the ratio of hyperbolic sines.[29] The latter probably increases with χ in an approximately monotonic fashion, so that if one had the equilibrium ^{13}C isotope effect for the dehydrogenation of formate (χ = 1.00) χ could be estimated from k_{12}/k_{13}. Unfortunately the equilbrium isotope effect is not known. However a value around 1.07 would be consistent with a variety of kinetic isotope effects on decarboxylation reactions[30] and with the present results, assuming a value of 0.4 for χ.

The reactant formate, in this case, is drawn from a pool that is 50% formic acid. The ^{13}C isotope effect on the ionization of formic acid does not seem to be known, but the equilibrium ^{13}C isotope effect on the ionization of benzoic acid is small,[31] and the ^{13}C kinetic isotope on decarboxyation reactions does not seem to change noticeably on ionization.[30]

The enzymatic reaction with the natural cofactor is considerably faster than the reaction with 10-methylacridinium ion,[3] and k_H/k_D is smaller, both of which would suggest that χ is even smaller in this case. However, k_{12}/k_{13} is 1.042, suggesting a higher value of χ, ~0.6, and the ^{18}O isotope effect also suggests a high value of χ.[3] In the nonenzymatic reaction, the various structural changes leading from reactant to product are probably well coordinated, because getting them out of phase would increase the molar energy without any compensation. In the enzymatic reaction, however, the structural changes around carbon, and, particularly, the desolvation of the carboxylate ion, may be much more advanced than the covalency changes around hydrogen if this stabilizes the protein. Such a desolvation of the formate would help to make the covalency changes more spontaneous. Cleland and coworkers have suggested that such desolvation occurs.[3] If this is true, it is no longer possible to locate the TS unambiguously on a two-dimensional map and χ

loses its simple significance. It is small with respect to the bonds to H, but large with respect to the environment of the carboxylate group.

It is a corollary of these ideas that the ^{13}C fractionation factor[32] for distribution of formate between water and non-hydrogen bond donor solvents should be appreciably less than 1.00. In fact, Cleland and coworkers have observed such a fractionation factor, 0.998, for the distribution of tetrabutylammonium formate between water and chloroform,[33] though this is a somewhat smaller effect than we would have anticipated. The change in the ^{13}C kinetic isotope effect on the oxidation of formate in dimethylsulfoxide-water mixtures, from 1.0154 in pure dimethylsulfoxide to 1.0362 in pure water,[3] is also consistent with this idea. Cleland and coworkers have attributed this change to a change in $\underline{X}$, but $\underline{X}$ would have to change from 0.5 to 0.2 to entirely account for the change in $\underline{k}_{12}/\underline{k}_{13}$. This seems like a very large change in $\underline{X}$, even though it accompanies a decrease in rate constant of a factor of 10^7. We suggest that the change in $\underline{k}_{12}/\underline{k}_{13}$ is partly due to a decrease in the ^{13}C fractionation factor of the reactant formate in the dimethylsulfoxide-rich solvents.

We doubt that the changes in isotope effects observed in the enzymatic reactions with unnatural cofactors are due to changes in $\underline{X}$, induced by the relative electron affinities of the 3-substituents, through changes in the spontaneity of the reactions. These effects would be too small to account for the observation.[6,11] Rather, we believe, the changes reflect the slightly poorer fit of the unnatural TSs in the reactive site of the protein. The fact that the isotope effects measured with the unnatural cofactors approach those for the nonenzymatic reaction supports this view.

References

1a. This work was supported by the U.S. National Science Foundation through Grant CHE-8215014, to the University of Minnesota.

b. Department of Chemistry, Buena Vista College, Storm Lake, Iowa 50588, U.S.A.

c. Department of Chemistry, University of Minnesota, 207 Pleasant St. S.E., Minneapolis, MN 55455, U.S.A.

2. White, A.; Handler, P.; Smith, E.L. "Principles of Biochemistry," 4th edition, McGraw Hill Book Co., N.Y., 1968, p. 359.

3. Hermes, J.D.; Morrical, S.W.; O'Leary, M.H.; Cleland, W.W. Biochemistry, 1984, 23, 5479.

4. Blanchard, J.S.; Cleland, W.W. Biochemistry, 1980, 19, 3543.

5. Cleland, W.W., Private communication.

6. Ostović, D.; Lee, I-S.H.; Roberts, R.M.G.; Kreevoy, M.M. J. Org. Chem., in press.

7. Clark, J.; Bakavoli, M. J. Chem. Soc. Perkin I, 1977, 1966.

8. Marcus, R.A., J. Phys. Chem., 1968, 72, 891.

9. Hassid, A.I.; Kreevoy, M.M.; Liang, T-M., Faraday Symp. Chem. Soc., 1975, 10, 69.

10. Albery, W.J. Annu. Rev. Phys. Chem., 1980, 31, 227.

11. Roberts, R.M.G.; Ostović, D.; Kreevoy, M.M., Faraday Discuss. Chem. Soc., 1982, 72, 257-265.

12. Ostović, D.; Roberts, R.M.G.; and Kreevoy, M.M., J. Am. Chem. Soc., 1983, 105, 7629.

13. Kreevoy, M.M.; Lee, I-S.H., J. Am. Chem. Soc., 1984, 106, 2550.

14. Powell, M.F.; Bruice, T.C., J. Am. Chem. Soc., 1983, 105, 7139.

15. Frost, A.A., Pearson, R.G., "Kinetics and Mechanism," 2nd edition, Wiley, N.Y. 1961; p. 29.

16. Moelwyn-Hughes, E.A.; Johnson, P. Trans. Faraday Soc., 1940, 36, 949.

17. Gold, V., "pH Measurements, Their Theory and Practice," Methuen and Co., Ltd., London, 1956, pp. 68-80.

18. O'Leary, M.H., Methods in Enzymology, 1980, 64, 83.

19. We are very much indebted to Drs. M.H. O'Leary and I. Triechel, Dept. of Chemistry, University of Wisconsin, Madison, for the use of their equipment and their generous assistance with these measurements.

20. Craig, H., Geochim. Cosmochim. Acta, 1957, 12, 133.

21. Selby, I.A. "Heterocyclic Compounds," vol. 9, 2nd edition, Butterworths, Ltd., London, 1970, p. 437.

22. Kolthoff, I.M.; Chantooni, M.K. Jr., J. Phys. Chem., 1962, 66, 1675; J. Am. Chem. Soc., 1963, 85, 426.

23. Sinha, A.; Bruice, T.C., J. Am. Chem. Soc., 1984, 106, 7291.

24. Carlson, B.W.; Miller, L.L., J. Am. Chem. Soc., 1985, 107, 479.

25. Carlson, B.W.; Miller, L.L.; Neta, P.; Gradkowski, J., J. Am. Chem. Soc., 1984, 106, 7233.

26. Loach, P.A., "Handbook of Biochemistry," Sabor, H.A., editor, CRC Press, Cleveland, Ohio, 1970, J-33.

27. Ostović, D., Ph.D. Thesis, University of Minnesota, 1985, p. 89.

28. Melander, L.; Saunders, W. H. Jr., "Reaction Rates of Isotopic Molecules," John Wiley and Sons, New York, N.Y., 1980, p. 45.

29. Eyring, H.; Cagle, F.W. Jr. J. Phys. Chem., 1952, 56, 889.

30. Dunn, G.E. "Isotopes in Organic Chemistry," vol. 3, Buncel, E. and Lee, C.C., editors, Elsevier, Amsterdam, 1977, Chapt. 1.

31. Bayles, J.W.; Bron, J.; Paul, S.O., J.C.S. Faraday I, 1976, 72, 1546.

32. Ref. 28, pp. 237-239.

33. Calculated from data given in Table IX of ref. 3.