Intermolecular Hydrogen Transfer Catalyzed by a Flavodehydrogenase, Bakers’ Yeast Flavocytochrome $b_2$*

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Bakers' yeast flavocytochrome $b_2$ is a flavin-dependent L-2-hydroxy acid dehydrogenase which also exhibits transhydrogenase activity. When a reaction takes place between [2-$^3$H]lactate and a halogenopyruvate, tritium is found in water and at the halogenopyruvate position. When the halogenopyruvate undergoes halide ion elimination, tritium is also found at the C3 position of the resulting pyruvate. The amount of this intermolecular tritium transfer depends on the initial keto acid-acceptor concentration. At infinite acceptor concentration, extrapolation yields a maximal transfer of 97 ± 11%. This indicates that the hydroxy acid-derived hydrogen resides transiently on the acceptor molecules. Exchange studies with bulk solvent, however, show only at the level of free reduced enzyme. Using a minimal kinetic scheme, the rate constant for hydrogen exchange between $E_{red}$ and $E_{ox}$ is calculated to be on the order of $10^5$ M$^{-1}$ s$^{-1}$, which leads to an estimated $pK$ ≈ 15 for the ionization of the substrate-derived proton while on the enzyme. It is suggested that this hydrogen could be shared between the active site base and $E_{red}$ N5 anion. It is furthermore shown that some tritium is incorporated into the products when the transhydrogenation is carried out in tritiated water. Finally, with [2-$^3$H]lactate-reduced enzyme, a deuteron isotope effect is observed on the rate of bromopyruvate disappearance. Extrapolation to infinite bromopyruvate concentration yields $\nu V = 4.4$. An apparent inverse isotope effect is determined for bromide ion elimination. These results strengthen the idea that oxidoreduction and elimination pathways involve a common carbanionic intermediate.

A number of flavoenzymes catalyze dehydrogenation reactions of the type

$\begin{align*}
\text{H} & \text{O} \\
\text{H} & \text{O} \\
(E, \text{Fl}_{ux}) + & -C-& -C- = (E, \text{Fl}_{red}) + -C-& -C- \\
\text{X} & \text{H} \\
\text{X} & \text{H}
\end{align*}$

The most generally accepted mechanism for such a reaction involves initial abstraction of the carbon-bound proton, with formation of a transient carbanion (1–3). There is, however, still room for debate. Furthermore, the nature of the electron transfer step(s) from the carbanion to oxidized flavin is still a moot point. Single electron transfer has been advocated (2) and covalent catalysis was demonstrated for the lactate oxidase-catalyzed glycolate oxidation (4, 5) and for nitroethane oxidation by D-amino acid oxidase (10).

One of the first pieces of evidence interpretable in terms of carbanion formation was the halide ion elimination from 3-chloroamino acids and 3-chlorolactate catalyzed by D-amino acid oxidase (6–11) and lactate oxidase from Mycobacterium smegmatis (11, 12), respectively. We have recently provided evidence that the reaction between halogenosubstrates and bakers' yeast lactate dehydrogenase (flavocytochrome $b_2$) is also interpretable in terms of a carbanion mechanism (13, 14). Our kinetic results strongly supported the hypothesis that halide ion elimination took place from an intermediate on the normal oxidoreduction pathway; they ruled out that elimination occurred during an irrelevant reaction between reduced flavin and oxidized halogenosubstrate, a possibility which has been discussed at length (10).

It should be recalled at this point that flavocytochrome $b_2$ from bakers' yeast is a bifunctional, tetrameric enzyme ($M_r = 4 \times 58,000$) carrying one FMN and one protoheme IX on each subunit (16). The electrons picked up from lactate by flavin are transferred one by one to heme $b_2$ and then to the external electron acceptor (17). The enzyme is a typical dehydrogenase/electron transferase (18), but we have shown that under transhydrogenation conditions the reduced heme is by-passed so that the enzyme functions as a simple flavodehydrogenase (15).

The fundamental observation presented in the present paper is that, when flavocytochrome $b_2$ functions in the transhydrogenation mode (19), it catalyzes an intermolecular hydrogen transfer from C2 of the hydroxy substrate to C2 of the keto substrate; for example the reduction of a halogeno-keto acid by [2-$^3$H]lactate-reduced enzyme yields a [2-$^3$H]halogenohydroxy acid, besides tritiated water. We believe the quantitative aspects of the results are such as to rule out mechanisms other than the carbanion mechanism for the dehydrogenation reaction. Furthermore, they enable us to make a reasonable guess as to which heteroatom the transferred proton is bound to while it resides on the protein.

Parts of this work have been presented previously in preliminary form (13, 19, 20).

**EXPERIMENTAL PROCEDURES**

**Materials**

Flavocytochrome $b_2$ was prepared from lyophilized commercial baker's yeast as described in Ref. 21. Enzyme concentration was expressed relative to the heme content using $d_{280}^\text{M} = 129.5$ mm$^{-1}$ cm$^{-1}$ or $d_{280}^\text{M} = 183.0$ mm$^{-1}$ cm$^{-1}$.$^{1}$ Enzymatic assays were performed at 30 °C in the presence of 20 mM L-lactate, 1 mM potassium ferricyanide in the standard buffer (0.1 M phosphate, 1 mM EDTA, pH 7.0).

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enzyme was stored at 4 °C as a 70% ammonium sulfate precipitate in the standard buffer containing 1 mM methimethylylauonil fluoride. Working solutions were prepared as described in Ref. 22. All commercial chemicals were used as such except for 3-chlorolactic acid which was purified according to Ref. 15. Beef heart lactate dehydrogenase came from Boehringer Mannheim, tritiated sodium borohydride (200 mCi/mmol) from the Centre d'Études Nucléaires (Saclay). L-[2-3H]Lactate was kindly given by Dr. D. Pompon, Centre National de la Recherche Scientifique (23).

**Methods**

**Preparation of Tritiated Hydroxy Acids—DL-[2-3H]Lactate** was prepared by chemical reduction of pyruvate. To 30 μmol of Na pyruvate in 0.9 ml of 0.1 M P1 buffer, pH 8.0, were added 62 μmol of NaBrO3 in 0.6 ml of 1 M NaOH, which was added to water to remove tritiated water, then with a 400-ml gradient of formic acid (0-2 M). The fractions containing pure DL-[2-3H]lactate were freed from formic acid by rotary evaporation after an overnight vacuum and dilution to a final volume of about 1 ml with water before neutralization with solid NaHCO3. The final yield was 96% and the specific radioactivity was 21,700 cpm nmol-1.

1-[2-3H]Halogenolactates were synthesized enzymatically. NAD+ (12.4 mg in 0.6 ml of 40 mM P1 buffer, pH 7.0) was mixed with DL-bromolactate (prepared from bromopyruvate as described in Ref. 15; 90 μmol in 0.5 ml of a neutral aqueous solution) or DL-chlorolactate (80 μmol in 0.5 ml of a neutral aqueous solution); 455 nmol of DL-[2-3H]lactate (9.9 × 105 cpm) in 0.35 ml of a neutral aqueous solution and 5 mg of beef heart lactate dehydrogenase in 0.1 ml standard buffer were then added. After 5 min at 31 °C, the reaction was quenched with concentrated HCl. The acidified mixture was applied to a column (0.9 × 16 cm) of AG 1-X8 (OH-) which was developed as described above. The specific radioactivities for L-bromo- and L-chloro-[2-3H]lactate were 68,600 and 53,400 cpm μmol-1, respectively.

**Analytical Methods**—The total 2-keto acid concentration in reaction mixtures or column effluents was determined by titration with excess NADH in the presence of beef heart lactate dehydrogenase as described in Ref. 15. Similarly, the 2-hydroxy acid concentration was determined by measuring the amount of ferricyanide reduced in the presence of flavocytochrome b5 (15). Bromopyruvate (Sigma) was assayed with the 5-thio-2-nitrobenzoate diazon (24).

**Incubation Conditions for Transhydrogenation Reactions—** All experiments were carried out with the cleaved form of the enzyme (16, 17) in standard buffer, in the dark and at 30 °C. The reaction mixture was incubated in a vial, stoppered with an air-tight silicon septum (typical a vial for the Waters Associates automatic injector WISP). The solutions were deoxygenated by blowing over the surface a gentle stream of purified argon (O2 < 2 ppm) for 10–15 min before beginning the reaction, with manual shaking. Reagents were introduced or aliquots removed through the septum by means of a Hamilton syringe (15). The total reaction volume was usually around 1 ml, and the reaction was quenched with 20 μl of concentrated HCl.

**Separation of Reaction Products—** The keto and hydroxy acids present at the end of transhydrogenation reactions were separated on a column of AG 1-X8 as described in Ref. 15 except that after loading, the column was first washed with 25 ml of H2O in order to elute specifically all the tritiated water before applying the formic acid gradient.

**Determination of Tritium Labeling Stereospecificity—** The 2-hydroxy acid isolated after AG 1-X8 chromatography was neutralized with solid NaHCO3 and the fraction was dried under vacuum. The solid residue was taken up in 1.5 ml of standard buffer and oxidized with flavocytochrome b5 in the presence of excess ferricyanide. When the reaction had reached completion, the mixture was acidified with 5 μl of concentrated HCl and loaded onto a column (0.6 × 5 cm) of AG 1-X8 eluted with water. The radioactive spots found in the effluent, namely tritiated water, was considered to originate from the 2R-position of the tritiated hydroxy acid.

**Results**

Before presenting the results, it should be recalled that, during transhydrogenation reactions catalyzed by flavocytochrome b5, chloropyruvate, and bromopyruvate, besides undergoing reduction to the corresponding halogenohydroxy acid, were shown to also undergo halogen elimination, thus giving rise to pyruvate. On the other hand, fluoropyruvate gave rise exclusively to fluorolactate (14).

**Hydrogen Transfer from Hydroxy Acid C2 to Keto Substrate C2**—Fig. 1 shows the separation of reactants and products in a typical transhydrogenation experiment between [2-3H]lactate and fluoropyruvate. As expected, tritium was found in residual lactate, and in water (eluted ahead of the formic acid gradient, see legend) and none was present in the residual fluoropyruvate or the lactate-derived pyruvate. But a sizeable amount of radioactivity was found in the product fluorolactate. Table I gives quantitative details about a similar experiment as well as about transhydrogenation reactions carried out between [2-3H]lactate and chloro- or bromopyruvates. All these reactions gave rise to intermolecular tritium transfer. The labeled bromolactate arising from such a reaction was incubated with flavocytochrome b5 in the presence of excess ferricyanide, so as to be oxidized back to bromopyruvate (see "Methods"). After the reaction, 97% of the bromolactate radioactivity was found as tritiated water. Thus, the label had been stereospecifically incorporated into the 2R-position. This showed the enzymatic nature of the tritium transfer; it was further attested by the fact that no transfer was observed in control experiments where active enzyme was replaced by flavin-free enzyme or by free FMN.

In other experiments (not shown), transhydrogenation was carried out between similar concentrations of fluoropyruvate and nearly saturating concentrations of either [2-3H]bromolactate or [2-3H]chlorolactate. Similar amounts of tritium transfer were observed, showing that the process was independent of the nature of the halogen on the isotope donor C3.

**Hydrogen Transfer from Hydroxy Acid C2 to Keto Substrate C3**—Fig. 2 illustrates the fact that when bromopyruvate was
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**Table 1**

| Substrate   | Initial concentration | Specific radioactivity | Radioactivity found in | Specific radioactivity of | \( r^2 \) |
|-------------|-----------------------|------------------------|-------------------------|--------------------------|-----------|
| Fluoropyruvate | 34.1 mM (3840 cpm pmol\(^{-1}\)) | 28.7 (273,000 cpm pmol\(^{-1}\)) | H\(_2\)O: 126,850, Halogenolactate: 80,480, Pyruvate: 30,460 | H\(_2\)O: 3 (8,700 cpm pmol\(^{-1}\)), Halogenolactate: 8 (2,700 cpm pmol\(^{-1}\)), Pyruvate: 0.048 | 0.388 |
| Chloropyruvate | 43.0 mM (3840 cpm pmol\(^{-1}\)) | 51.0 (294,000 cpm pmol\(^{-1}\)) | H\(_2\)O: 226,600, Halogenolactate: 284,600, Pyruvate: 700 | H\(_2\)O: 5 (10,400 cpm pmol\(^{-1}\)), Halogenolactate: 1,750 (0.57), Pyruvate: 0.048 | 0.388 |
| Bromopyruvate | 7.5 mM (3840 cpm pmol\(^{-1}\)) | 25.7 (276,000 cpm pmol\(^{-1}\)) | H\(_2\)O: 3,075,000, Halogenolactate: 119,935, Pyruvate: 33,830 | H\(_2\)O: 14 (13,200 cpm pmol\(^{-1}\)), Halogenolactate: 2,700 (0.048), Pyruvate: 0.048 | 0.388 |

*\( r^2 \) is the ratio of the sum of radioactivity found in the halogenohydroxy acid and pyruvate to the total radioactivity transferred from [\( ^3\)H]lactate (found in water, halogenohydroxyacid and, if any, in pyruvate).

† Enzyme concentration was 15 \( \mu \)M. The reaction was stopped after 20 min by freezing.

‡ Enzyme concentration was 12 \( \mu \)M. The reaction was quenched after 45 min by freezing.

§ This figure takes into account only the pyruvate produced by halide ion elimination from the halogenopyruvate.

It is not corrected for the slow isotope exchange with the medium (see text).

†† For detailed experimental conditions, see Fig. 2.

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**FIG. 2. Transhydrogenation reaction between [2-\( ^3\)H]lactate and bromopyruvate: separation of reactants and products.**

The initial conditions were 25.7 mM [\( ^3\)H]lactate (276,000 cpm pmol\(^{-1}\)), 7.5 mM bromopyruvate, and 10.3 \( \mu \)M flavocytochrome \( b_2 \) in 1.0 ml standard buffer. The following anaerobic additions were made: 7.6 \( \mu \)mol of bromopyruvate and 2.3 nmol of enzyme after 6 min; 6.9 \( \mu \)mol of lactate, 11.4 \( \mu \)mol of bromopyruvate, and 4.6 nmol of enzyme after another 12 min; 2.3 nmol of enzyme after another 6 min. The reaction was stopped after 30 min incubation altogether. This multistep procedure avoided high bromopyruvate concentrations responsible for bromolactate, bromopyruvic acids, and bromopyruvic anhydride. Several controls were run in order to find out whether the isotope incorporation into pyruvate is enzyme-catalyzed and therefore that flavocytochrome \( b_2 \) can transfer the proton abstracted from lactate to the keto substrate C3. Table I shows that in each experiment the pyruvate specific radioactivity was lower than that of the halogenopyruvate. For detailed experimental conditions, see Fig. 2.

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**Table 1**

**Tritium transfer between [2-\( ^3\)H]lactate and various halogenopyruvates**

| Substrate   | Initial concentration | Specific radioactivity | Radioactivity found in | Specific radioactivity of | \( r^2 \) |
|-------------|-----------------------|------------------------|-------------------------|--------------------------|-----------|
| Fluoropyruvate | 34.1 mM (3840 cpm pmol\(^{-1}\)) | 28.7 (273,000 cpm pmol\(^{-1}\)) | H\(_2\)O: 126,850, Halogenolactate: 80,480, Pyruvate: 30,460 | H\(_2\)O: 3 (8,700 cpm pmol\(^{-1}\)), Halogenolactate: 8 (2,700 cpm pmol\(^{-1}\)), Pyruvate: 0.048 | 0.388 |
| Chloropyruvate | 43.0 mM (3840 cpm pmol\(^{-1}\)) | 51.0 (294,000 cpm pmol\(^{-1}\)) | H\(_2\)O: 226,600, Halogenolactate: 284,600, Pyruvate: 700 | H\(_2\)O: 5 (10,400 cpm pmol\(^{-1}\)), Halogenolactate: 1,750 (0.57), Pyruvate: 0.048 | 0.388 |
| Bromopyruvate | 7.5 mM (3840 cpm pmol\(^{-1}\)) | 25.7 (276,000 cpm pmol\(^{-1}\)) | H\(_2\)O: 3,075,000, Halogenolactate: 119,935, Pyruvate: 33,830 | H\(_2\)O: 14 (13,200 cpm pmol\(^{-1}\)), Halogenolactate: 2,700 (0.048), Pyruvate: 0.048 | 0.388 |

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**Incorporation of Tritium from Tritiated Water—Transhydrogenation reactions were carried out in tritiated water, using lactate and relatively low concentrations of bromopyruvate, so as to be under conditions where little intermolecular hydrogen transfer would take place.**

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**Discussion**

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**Elution volume (ml)**

**Keto acids ([\( ^3\)H] and halogenoacids) (µmol)**

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**Fig. 2. Transhydrogenation reaction between [2-\( ^3\)H]lactate and bromopyruvate: separation of reactants and products.**

The initial conditions were 25.7 mM [\( ^3\)H]lactate (276,000 cpm pmol\(^{-1}\)), 7.5 mM bromopyruvate, and 10.3 \( \mu \)M flavocytochrome \( b_2 \) in 1.0 ml standard buffer. The following anaerobic additions were made: 7.6 \( \mu \)mol of bromopyruvate and 2.3 nmol of enzyme after 6 min; 6.9 \( \mu \)mol of lactate, 11.4 \( \mu \)mol of bromopyruvate, and 4.6 nmol of enzyme after another 12 min; 2.3 nmol of enzyme after another 6 min. The reaction was stopped after 30 min incubation altogether. This multistep procedure avoided high bromopyruvate concentrations responsible for slow enzyme inactivation of free bromolactate, bromopyruvic acids, and bromopyruvic anhydride. Several controls were run in order to find out whether the isotope incorporation into pyruvate is enzyme-catalyzed and therefore that flavocytochrome \( b_2 \) can transfer the proton abstracted from lactate to the keto substrate C3. Table I shows that in each experiment the pyruvate specific radioactivity was lower than that of the halogenopyruvate. For detailed experimental conditions, see Fig. 2.
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of about 2 (approximately 11 column volumes). For the sake of activity level in the effluent had fallen to a signal/background ratio column. The initial elution with water was prolonged until the radioactive enzyme, and 11.4 pmol of bromopyruvate. After another 5 min, 2.0 nmol of enzyme were again added and the reaction was stopped after 6 min, 7.7 pmol of bromopyruvate and 2.0 nmol of enzyme were again added and the reaction was stopped after a total of 28 min. The mixture was separated on the usual AG 1-X8 column. The initial conditions were 25 mM L-lactate, 7.7 mM bromopyruvate, and 10 µM flavocytochrome b. The reaction was stopped by freezing after a 20-min incubation. The mixture was acidified before loading onto the usual column (0.9 cm X 16 cm) of AG 1-X8. The elution procedure was modified so as to improve the separation between lactic and fluorolactic acids; the column was first washed with 25 ml of H2O, which removed untritiated water, then with a gradient between 0 and 1.33 M formic acid (400 ml total volume).

![Figure 3: Influence of the initial fluoropyruvate concentration on the amount of tritium transfer (τ).](image)

![Figure 4: Transhydrogenation in tritiated water: separation of reactants and products.](image)

It can be concluded that lactate carried tritium at the C2 and at the C3 positions. The most likely explanation is that, as tritiated pyruvate built up during incubation, it started undergoing reduction in competition with bromopyruvate. In view of the figures, it is thus impossible to decide whether any C2 tritium could have been picked up by reprotonation of the lactate-derived carbanion.

The specific radioactivity of bromolactate in these experiments was between 24- and 30-fold lower than that of tritiated water. These figures cannot, however, be considered as intrinsic isotope discrimination factors. Indeed, since unlabeled lactate was used, discrimination against tritium could occur at two points in the overall solvent isotope incorporation, namely when the enzyme exchanged the lactate-derived proton, and when it protonated the carbanion to yield bromolactate. Therefore the observed figure is a combination of two effects.

Deuterium Kinetic Isotope Effect on Bromopyruvate Transformation—Since α-hydrogen abstraction is rate-determining in the 2-hydroxy acid oxidation (forward reaction (15, 23)), the principle of microscopic reversibility wants carbon-hydrogen bond formation to be rate-determining in the reverse reaction (14, 15). The slow isotope exchange between reduced enzyme and solvent offered a means of verifying this conclusion. With [2-3H]lactate as reducing substrate, one would expect to observe a kinetic isotope effect on halogenolactate formation; furthermore the isotope effect should be dependent on the extent of enzyme deuteration, in other words, on halogenopyruvate concentration. We determined the disappearance rate of bromopyruvate at various concentrations in the presence of saturating concentrations of either [2-1H] lactate or [2-3H]lactate. Fig. 5 shows a double reciprocal plot which allowed an extrapolation to infinite bromopyruvate concentration. A value of 4.4 ± 0.4 could thus be determined for 3V. It should be stressed that with deutolactate, the enzyme reduction rate is still about 15 times faster than bromopyruvate disappearance at the maximum concentration used (14, 23) and could not become a limiting factor.

It has been explained previously that the rate of pyruvate formation (elimination) from a halogenopyruvate in a transhydrogenation reaction can be monitored by determining the rate of total keto acid concentration increase (14). Using this method we determined the deuterium isotope effect on bromide ion elimination. A complete study like that of Fig. 5 could not be carried out because the bromopyruvate concentration range that could be conveniently explored was limited.
both on the low and the high side (14). The results are presented in Table III. It can be seen that an apparent inverse isotope effect was observed. This fact, which is at first sight surprising, can be easily explained if one looks at Scheme 1. (Scheme 3 of Ref. 14). According to our previous qualitative analysis of this scheme, the rate-determining step in pyruvate formation is actually halide ion departure (step 5). Therefore $k_5$ itself should not be subject to an isotope effect. But, according to Scheme 1, since bromolactate formation (step 2) is subject to an isotope effect, when the enzyme is deuterated more carbanion will be available for elimination and the rate of this reaction will consequently increase as was observed. A similar observation of an inverse isotope effect in one of two reactions competing for the same intermediate has been reported (25).

The $k_{\text{cat}}/K_m$ ratio for bromolactate formation is only about 2-fold larger than for elimination (14). Since $^2\text{H}$ deduced from Fig. 5 applies to bromopyruvate disappearance (in other words to the sum of reduction and elimination), it can be concluded that the figure of 4.4 is only a lower estimate of the actual $^2\text{H}$ for reduction.

**DISCUSSION**

The phenomenon of intermolecular hydrogen transfer documented in what precedes shows the substrate-derived α-hydrogen to be exchanged only slowly with solvent by reduced flavocytochrome $b_5$. Furthermore, it has been shown that this hydrogen, while on the enzyme, resides on a monoprotic heteroatom.

**Examples of Slow Hydrogen Exchange in Other Flavoproteins**—Slow hydrogen exchange with solvent has indeed been described in a number of cases. Direct hydrogen isotope transfer from substrate C2 to substrate C3 was demonstrated during elimination from chlorolactate by lactate oxidase (12) and from β-chloroamino acids by D-amino acid oxidase (8, 26). Lack of exchange with solvent was also observed in the transfer of the substrate hydrogen from the C2 to the C4 position during the decarboxylation catalyzed by glutaryl-CoA dehydrogenase (27). Kinetic evidence for a deuterium isotope effect on the reverse reaction rate of lactate oxidase with glycollate (5), of D-amino acid oxidase with various substrates (28) and D-lactate dehydrogenase from *Megasphaera elsdenii* (29) also pointed to the absence of hydrogen exchange during the lifetime of the enzyme-intermediate(s) complex. Similar conclusions were drawn from the study of proton release timing during catalysis by D-amino acid oxidase (30) and in the complexation of lactate oxidase with oxalate, during which an active site group was found to undergo a $pK$ raise from 4.9 to 9.8 (31). The important point is that in all those cases the proton or its isotope was given back to the same substrate molecule it had been picked up from and it is the enzyme-ligand complex that exchanged a critical proton very slowly if at all. Therefore those cases are examples of intramolecular transfers and it can be surmised that the proton was physically shielded from the solvent by the ligand.

Gilbert (32) has discussed at length the catalytic advantage of excluding bulk solvent from the active site upon substrate binding when the reaction catalyzed is proton transfer to or from a carbon acid. It thus appears that desolvation plays an important role in flavodehydrogenase catalysis.

Actual cases of intermolecular transfer where the hydrogen should be available to solvent after product dissociation and before binding of another substrate molecule are much rarer for natural flavoproteins. Bacterial and beef heart mitochondrial nicotinamide nucleotide transhydrogenases (33, 35), and NADH cytochrome $b_5$ reductase (36, 37) were shown to catalyze isotope transfer between two different nicotinamide molecules. On the other hand, mitochondrial NADH-cytochrome $c$ reductase (38), D-amino acid oxidase (6), and glutamate synthase (39) reportedly failed to catalyze intermolecular hydrogen transfer. Several papers described intermolecular transfers catalyzed by enzymes reconstituted with 5-deazaflavin (40–46). But the C5 position of the analog is nonexchangeable and, since it is generally assumed that 5-deaza-

**Table III**

**Deuterium isotope effect on elimination from bromopyruvate**

| Experiment | Isotope transferred | Bromopyruvate concentration (mM) | $k_{\text{cat}}$ (pyruvate formation) | $\delta k_{\text{cat}}$ |
|------------|---------------------|----------------------------------|--------------------------------------|---------------------|
| 1          | $^2\text{H}$        | 6.70                             | 0.47                                 | 0.50                |
|            | $^2\text{H}$        | 6.30                             | 0.33                                 | 0.50                |
| 2          | $^2\text{H}$        | 8.50                             | 0.56                                 | 0.51                |
|            | $^2\text{H}$        | 8.35                             | 1.09                                 | 0.51                |
| 3          | $^2\text{H}$        | 8.60                             | 0.69                                 | 0.60                |
|            | $^2\text{H}$        | 8.30                             | 1.14                                 | 0.60                |
flavin is a nicotinamide analog and functions by hydride transfer (18), these cases are relevant for this discussion only insofar as they point to the geometrical feasibility of direct hydrogen transfer from substrate to cofactor. In conclusion, the intermolecular hydrogen transfer observed with flavocytochrome b is the first example of a non-nicotinamide-linked flavoenzyme-catalyzed intermolecular transfer.

Mechanistic Consequences of the Flavocytochrome b-catalyzed Intermolecular Hydrogen Transfer—We have previously discussed the evidence leading to Scheme 1 as the best rationalization of our findings in the study of flavocytochrome b-catalyzed oxidation, reduction, and elimination reactions. The results reported in this paper bring additional support in favor of Scheme 1.

First, the existence of a deuterium isotope effect on the rate of bromopyruvate disappearance confirms that, in the reduction of keto acids, αC—H bond formation is governed by the rate-determining breaking of an enzyme X—H bond. As discussed under "Results," the experimental value of 4.4±0.4 is a lower estimate of the intrinsic isotope effect on that step. Let us recall that a δν = 7–8 was obtained for flavin reduction by lactate at 4 °C (23) and a similar value is probable for reduction by bromolactate, since similar figures were obtained for the overall transfer to ferriyanide from bromolactate and lactate (4ν = 4.7 at 30 °C (15) and 4ν = 5 at 30 °C (47) and 4 °C (23), respectively).

Second, the existence of an apparent inverse isotope effect on elimination strongly supports the idea that a central carboxylic anion can undergo the two competing reactions of protonation and elimination, the former (step 2) being subject to a strong isotope effect and the latter (step 5) being independent of isotope substitution. If elimination were occurring by hydride ion displacement of the halogen (48), one would expect an isotope effect on the elimination reaction.

Third, in transhydrogenation experiments between [2-2H]lactate and bromopyruvate or chloropyruvate, the specific radioactivity of the pyruvate formed by elimination was found to be significantly lower than that of the respective halogenolactates (Table I). This could have resulted from ketonization of some of the enol in the solvent after release from the protein. In the case of hydride or radical mechanisms, it is difficult to conceive how such a result could arise. Therefore these figures give additional support to a carbanion mechanism. Studies on D-amino acid oxidase-catalyzed elimination from 3-chloroamino acids also concluded that an initially formed enamine was isomerized to imino acid partly on the enzyme and partly in the solvent (8, 26).

Possible Identity of the Substrate-Proton Acceptor on the Protein—In order to try to evaluate the rate of proton exchange, let us consider Scheme 2, where KA and HA stand for keto acid and hydroxy acid, respectively, T for tritium, and B for buffer. Several assumptions are implicit in this scheme: (i) only the free reduced enzyme can exchange its isotope with the solvent; this is a reasonable assumption in view of the accumulated evidence for slow or nonexistent exchange in flavoenzyme-ligand complexes (5, 8, 12, 26, 29–31). In the flavocytochrome b case, it is furthermore supported by the data of Fig. 3, which indicate complete transfer, i.e. no exchange, at infinite substrate concentration, when no free reduced enzyme can be present. (ii) Enzyme reduction by HA, is supposed to be rapid compared to reduction by HA, so that during the observation time H-HA and T-HA formation can be considered as practically irreversible. This assumption appears reasonable for flavocytochrome b when HA, and HA, are lactate and fluorolactate, respectively (15); it was, however, verified by checking that, at 37.2 mM initial fluoropyruvate concentration, under experimental conditions similar to those of Fig. 3, the amount of transfer was the same after 11, 20, and 30 min incubation. (iii) The only isotope-sensitive step is supposed to be the one governed by k cat. (iv) Substrate-derived hydrogen and tritium atoms are supposed to reside only on monoprotic heteroatoms while on the enzyme.

With the definition of τ given in Table I:

\[ \tau = \frac{k_{\text{cat}}}{k_{\text{cat}} + k_{\text{cat}}} = \frac{1}{\frac{1}{k_{\text{cat}}} + \frac{1}{k_{\text{cat}}}} \]

using Scheme 2, one can demonstrate under steady-state conditions:

\[ \frac{1}{\tau} = \frac{1}{k_{\text{cat}}} \frac{(k_{\text{cat}} - k_{\text{cat}}) + k_{\text{cat}}}{k_{\text{cat}} - k_{\text{cat}}} + 1 \]

This equation predicts a linear relationship between 1/τ and the inverse of fluoropyruvate concentration, extrapolating to 1 at infinite fluoropyruvate concentration. This is what was experimentally obtained (Fig. 3). Besides indicating that the active site must be indeed monoprotic, this representation can yield a value for k cat, namely for the exchange rate constant under the given experimental conditions, provided that some knowledge concerning the other rate constants is available. The following values or estimates can be used. (i) Since k cat for fluoropyruvate reduction is very small (0.4 s⁻¹ (15)) we assume K D = K P. Its value (7.5 mM) being about 10-fold higher than the KD of lactate for Ecat, we take for fluoropyruvate k cat ≈ 10⁵ M⁻¹ s⁻¹ (similar to k cat for lactate) and k cat ≈ 10⁵ s⁻¹ (1 order of magnitude larger than k cat for lactate (23, 49–51)). (ii) By applying the Swain-Schaad relationship (52) to k cat, assuming that the actual value of k cat lies between 5 and 8, we obtain k cat ≈ 0.02–0.04 s⁻¹.

With these assumptions, we can derive from Equation 3:

\[ k_{\text{cat}} = \alpha \cdot k_{\text{cat}} \cdot k_{\text{cat}} \cdot k_{\text{cat}} K_{P} \]

where α is the slope of the double reciprocal plot. With α = 43 × 10⁻⁵ M⁻¹, one can calculate a value of 0.1–0.2 s⁻¹ for k cat, the pseudo-first order rate constant for tritium exchange in 0.1 M phosphate buffer, pH 7.0, at 30 °C. This value is very low. Could exchange itself be rapid but dependent on the slow reversal of a conformational change which would bury the protonated catalyst? This hypothesis would require that the reversal rate also govern the catalytic reaction rate. This is hardly tenable since with the various keto substrates, k cat values as high as 4 s⁻¹ were determined for the reduction reaction (15).
We shall then assume that the value of \( k' \) is a reasonable approximation of the rate constant for the actual exchange process. Since the active site is open to solvent, exchange will be buffer-catalyzed, hence
\[
k_r^* = k_r(\text{HPQ}) \approx 3-6 \text{ M}^{-1} \text{s}^{-1}
\]
and, assuming a maximal discrimination against tritium,
\[
k_r^b = 60-120 \text{ M}^{-1} \text{s}^{-1}
\]
This figure appears to be very low for the buffer-catalyzed exchange rate constant of a proton bound by a normal protein side chain. For an equilibrium
\[
DH + A^- \rightleftharpoons D^- + AH
\]
where \( D \) is the proton donor and \( A^- \) the acceptor, there is a simple relationship between the pK values of the exchanging species and the equilibrium rate constants (53, 54):
\[
\log k_r - \log k_r = pK_A - pK_D
\]
According to Eigen (54, 55), in the thermodynamically favorable direction, the rate constant will be diffusion limited, and hence have a value on the order of \( 10^6 \text{ M}^{-1} \text{s}^{-1} \). A protein nucleophile with a normal pK (3.5 < \( pK < 10 \)) should therefore present exchange rate constant values between 10 and 10^6 M^{-1} s^{-1}.

Using Equation 6 and the value of \( k_r^b \), a value of about 15 can be calculated for the pK of the exchanging species DH. This value is a very high one for a protein side chain, even though pK shifts have been determined in numerous cases (56, 57). The largest one reported so far is the decrease of 3.5 units upon binding of the substrate-derived proton between the active site base and of reduced flavin (64, 65).

Among the factors that effect the exchange rate of a proton and hence its pK, the most effective is hydrogen bonding, which lowers the rate in many cases by factors of 102-106, sometimes by much more (55, 62, 63). Very strong hydrogen bonding would have to be invoked for a raise of 7-8 units in the pK of a cysteine or a histidine, and of more for a carboxylic group. A hydrogen bond between the active site base and reduced flavin N5 anion would be expected to be very strong, since the N1 position is already supposed to be anionic in flavoproteins which bind sulfite (64, 65). An attractive situation, which may be mechanistically relevant, would be the sharing of the substrate-derived proton between the active site base and flavin N5, in an arrangement similar to that of the proton shared by two catalytic aspartates in aspartyl proteases (66). This doubly bonded hydrogen could have the pK of 15, a value intermediate between the pK values of the active site base and of reduced flavin N5—H.

Our preliminary results have shown lactate oxidase to also exchange the substrate-derived α-proton rather slowly (13, 20). On the other hand D-amino acid oxidase would exchange it rapidly (6). Among nicotinamide-linked dehydrogenases, exchange appears to proceed at rather diverse rates (33–37). Therefore the mechanistic significance of the flavin N5—H bond acidity remains to be assessed.

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