8-PhDS (Figure 7), strongly suggested that the PC and diacylglycerol analogues acted differently. We also observed recently that lyso-PC(oleoyl) activates PKC in the presence or absence of diacylglycerol, accompanied by an increased affinity for PS but a decreased affinity for Ca^{2+} (Oishi et al., 1988). These findings indicate that lyso-PC has mechanisms of action that are likely distinct from those for diacylglycerols and DS and DSPC analogues, further suggesting a complexity of PKC regulation by lipids. Introduction of branched chains to phospholipid and/or diacylglycerol would represent a new approach with which to investigate molecular events and specificity crucial for PKC activation and inactivation.

References

Cason, J., Wolfhagen, H. J., Tarpey, W., & Adams, R. E. (1949) *J. Org. Chem. 14*, 147–154.

Castagna, M., Akai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., & Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 7847–7851.

Charp, P. A., Rice, W. G., Raynor, R. L., Reimund, E., Kinkade, J. M., Jr., Ganz, T., Selsted, M. E., Lehrer, R. I., & Kuo, J. F. (1988a) *Biochem. Pharmacol. 37*, 951–956.

Charp, P. A., Zhou, Q., Wood, M. G., Jr., Raynor, R. L., Menger, F. M., & Kuo, J. F. (1988b) *Biochemistry 27*, 4607–4612.

Eibl, H., McIntyre, J. O., Fleer, E. A. M., & Fleischer, S. (1983) *Methods Enzymol. 98*, 623–632.

Fjuita, I., Irita, K., Takeshiga, K., & Minakami, S. (1984) *Biochem. Biophys. Res. Commun. 120*, 318–324.

Ganong, B. R., Loomis, C. R., Hannun, Y. A., & Bell, R. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1184–1188.

Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 12604–12609.

Helfman, D. M., Barnes, K. C., Kinkade, J. M., Jr., Vogler, W. R., Shoji, M., & Kuo, J. F. (1983) *Cancer Res. 43*, 2955–2961.

Kishimoto, A., Takai, Y., Mori, T., Kakkawa, U., & Nishizuka, Y. (1980) *J. Biol. Chem. 255*, 2273–2276.

Lapetina, E. G., Reep, B., Ganong, B. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 1358–1361.

Mazzei, G. J., Katoh, N., & Kuo, J. F. (1982) *Biochem. Biophys. Res. Commun. 109*, 1129–1133.

McPhail, L. C., Clayton, C. C., & Snyderman, R. (1984) *Science (Washington, D.C.) 224*, 622–625.

Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y., & Fujikura, T. (1982) *J. Biochem. (Tokyo) 91*, 427–431.

Nishizuka, Y. (1984) *Nature (London) 308*, 693–698.

Oishi, K., Raynor, R. L., Charp, P. A., & Kuo, J. F. (1988) *J. Biol. Chem. 263*, 6865–6871.

Post, R. L., & Sen, A. K. (1967) *Methods Enzymol. 10*, 773–775.

Sharkey, N. A., & Blumberg, P. M. (1985) *Cancer Res. 45*, 19–24.

Solaro, R. L., & Shiner, J. S. (1976) *Circ. Res. 39*, 8–14.

Su, H.-D., Mazzei, G. J., Vogler, W. R., & Kuo, J. F. (1985) *Biochem. Pharmacol. 34*, 3649–3653.

Turner, R. S., & Kuo, J. F. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J. F., Ed.) Vol. 2, pp 75–110, CRC, Boca Raton, FL.

Wise, B. C., Raynor, R. L., & Kuo, J. F. (1982) *J. Biol. Chem. 257*, 8481–8488.

Rat Kidney L-2-Hydroxyacid Oxidase. Structural and Mechanistic Comparison with Flavocytochrome b_2 from Baker's Yeast

Philippe Urban, Isabelle Chirat, and Florence Lederer*

Unité Associée au Centre National de la Recherche Scientifique UA 122 et Unité 25 de l'Institut National de la Santé et de la Recherche Médicale, Hôpital Necker, 75743 Paris Cédex 15, France

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ABSTRACT: Hydroxyacid oxidase from rat kidney is an FMN-dependent enzyme that catalyzes the oxidation of L-α-hydroxy acids as well as, more slowly, that of L-α-amino acids. We report here a modified purification method for the enzyme, which is found to possess one cofactor per subunit of M_r 39 000. Determination of its N-terminal sequence suggests the protein is homologous to spinach glycolate oxidase and baker's yeast lactate dehydrogenase. In the presence of a hydroxy acid and of bromopyruvate, under anaerobic conditions, the enzyme is found to catalyze both transhydrogenation and reductive bromide ion elimination. It had previously been observed that hydroxyacid oxidase could not catalyze chloride elimination from chlorolactate in the presence of oxygen [Cromartie, T. H., & Walsh, C. T. (1975) *Biochemistry 14*, 3482–3490]. The behavior of this enzyme toward halogeno substrates is therefore similar to that of baker's yeast L-lactate dehydrogenase and in part different from that of *Mycobacterium smegmatis* lactate oxidase and porcine kidney D-amino-acid oxidase. These findings can be rationalized on the basis of a common mechanism for all these enzymes, implying formation of a carbanion as a first step, with different rate-limiting steps in the overall reaction.

L-α-Hydroxyacid oxidase from rat kidney (EC 1.1.3.15) is an FMN-containing flavoenzyme that oxidizes L-α-hydroxy acids to keto acids with formation of hydrogen peroxide at the expense of oxygen (Blanchard et al., 1945, 1946). It was first isolated as an L-amino-acid oxidase (EC 1.4.3.2) (Blanchard et al., 1945) and subsequently shown to be more active with aliphatic longer chain homologues of glycolate, as well as with aromatic hydroxy acids such as mandelate and phenyllactate (Blanchard et al., 1946; Nakano & Danowski, 1966). More recently, Brush and Hamilton (1981) suggested that thiol–

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glyoxylate adducts were its true physiological substrates. The enzyme is located in peroxisomes (Tolbert, 1981; Angermüller et al., 1986 a). In rat kidney, it is the only α-hydroxy acid oxidizing activity present (Blanchard et al., 1946; Nakano & Danowski, 1966; McGroarty et al., 1974). In pig kidney peroxisomes, it is found together with glycolate oxidase (short-chain hydroxyacid oxidase, EC 1.1.3.1) (Robinson et al., 1962); only the latter is present in mammalian liver and leaf peroxisomes (Tolbert, 1981; Angermüller et al., 1986b).

Rat kidney hydroxyacid oxidase has been purified by various groups (Blanchard et al., 1945; Nakano & Danowski, 1966; Cromartie & Walsh, 1975a; Duley & Holmes, 1976). Cromartie and Walsh (1975b) carried out mechanistic studies on the enzyme in order to obtain evidence in favor of the so-called carbanion mechanism. Their observations were highly similar to those made earlier with L-lactate dehydrogenase from baker's yeast (flavocytochrome b_2) (Lederer, 1974): a strong deuterium isotope effect showed α-hydrogen abstraction to be the rate-limiting step in the steady state; the enzyme did not promote halide ion elimination from β-chlorolactate, even though it accepted the compound as a substrate; finally, 2-hydroxy-3-butynoate behaved as a suicide substrate for the enzyme.

Since then, we have found that, under appropriate conditions, flavocytochrome b_2 can catalyze halide ion elimination from β-halogeno substrates and have predicted that similar conditions would enable the observation of hydroxyacid oxidase catalyzed elimination (Urban & Lederer, 1984; Lederer, 1984). In this paper, we report experiments that verify our hypothesis. In addition, our characterization of the enzyme purified with a modified procedure solves a discrepancy found in the literature concerning the prosthetic group stoichiometry. Finally, N-terminal sequencing of rat kidney L-hydroxyacid oxidase suggests it to be homologous to glycolate oxidase (Volokita & Somerville, 1987) and flavocytochrome b_2 (Lederer et al., 1985; Guiard, 1985).

Part of the experiments reported in this paper have been previously presented in preliminary form (Lederer, 1984; Urban et al., 1987).

Materials and Methods

Chemicals

Sodium D,L-2-hydroxybutyrate, L-mandelic acid, and sodium 2,6-dichlorophenolindophenol came from Fluka; L-lactic acid, D,L-chlorolactic acid, and bromopyruvic acid came from Sigma; sodium D,L-2-hydroxyvalerate, D,L-2-hydroxycaproic acid, and L-2-hydroxyisocaproic acids were purchased from Aldrich. Commercial chlorolactic acid was freed from contaminating oxalate as described by Urban et al. (1983).

Methods

Protein concentrations were determined by the Bio-Rad protein microassay procedure using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of SDS[1] was carried out in a vertical flatbed apparatus as described by Douglas et al. (1979).

The tryptophan content of the protein was determined by fluorometry with a Perkin-Elmer LS-5 spectrofluorometer, using the internal calibration method of Pajot (1976). The FMN content of the purified protein was determined according to the same principle, with free FMN aliquots added to enzyme dissolved in 6 M guanidine hydrochloride. The fluorescence emission was recorded at 560 nm with excitation wavelength set at 450 nm. For both tryptophan and FMN determinations, the corresponding protein contents were estimated by amino acid analysis after protein hydrolysis.

S-Carboxymethylation with [1-^{14}C]iodoacetic acid was carried out in 6 M guanidine hydrochloride in the presence of dithiothreitol, following the procedure of Crestfield et al. (1963). Protein hydrolyses and amino acid analyses were performed as described (Lederer, et al., 1983). Edman degradations were carried out with an Applied Biosystems sequencer, Model 470A, equipped with online phenylthiohydantoin identification on the 120A analyzer.

Enzyme Purification. Rat kidneys from Wistar strain adult rats of mixed sex were used. They were washed in saline and frozen in liquid nitrogen after removal of the bulk of membranes and fat. They were thawed in the cold room overnight for enzyme extractions.

Purification was carried out by starting with 100 g of kidneys, at 0–4 °C for all steps. All buffers contained 1 mM $PheMeSO_2F$. The organs were homogenized in a Waring blendor in 400 mL of 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris–acetate buffer, pH 8. The homogenate was centrifuged at 16000*g* for 40 min. $PheMeSO_2F$ was added to the supernatant to a final concentration of 1 mM. The protein precipitating between 32% and 58% saturation of ammonium sulfate at pH 7.7 was collected by centrifugation and dissolved in the minimum volume of 5 mM phosphate buffer, 10 μM FMN, and 0.5 mM EDTA, pH 7.1. After dialysis against 2 × 2.4 L of the same buffer, some denatured protein was removed by centrifugation at 17000*g* for 1 h.

The supernatant was supplemented with 1 mM $PheMeSO_2F$, diluted if necessary to an ionic strength similar to that of the equilibrating buffer, and applied to a column of DEAE-cellulose (Whatman DE 52, 2.6 × 27 cm) equilibrated with 5 mM phosphate buffer and 0.5 mM EDTA, pH 7.0. The column was then washed with 0.35 L of the same buffer at a flow rate of 40 mL/h. It was then eluted at the same flow rate with a linear gradient between 500 mL of this buffer and 500 mL of 150 mM phosphate buffer and 1.5 mM EDTA, pH 8.0. The fractions containing hydroxyacid oxidase activity were concentrated by ammonium sulfate precipitation at pH 7.7. After centrifugation, the precipitate was dissolved in the minimal volume of 5 mM phosphate buffer, 10 μM FMN, and 0.5 mM EDTA, pH 7.1, and dialyzed against 2 × 2.4 L of the same buffer.

The solution was adsorbed onto a column of hydroxyapatite (Bio-Gel HTP from Bio-Rad, 2.6 × 23 cm) equilibrated with 10 mM phosphate buffer and 1 mM EDTA, pH 7.0. After being washed with 0.25 L of the same buffer at a flow rate of 35 mL/h, the column was eluted with a linear gradient between 800 mL of equilibrating buffer and 800 mL of 0.8 M phosphate buffer and 1.5 mM EDTA, pH 7.2, at a flow rate of 40 mL/h. The fractions showing activity were precipitated by addition of ammonium sulfate to 60% saturation and stored at 4 °C.

The standard activity assay solution consisted of 25 mM D,L-2-hydroxybutyrate, 70 μM DCIP (0.002%) in 0.1 M Tris-HCl buffer and 1 mM EDTA, pH 8.4. Dye reduction was monitored with a Cary 118 CX spectrophotometer at 30 °C. An extinction coefficient of 22000 M^{-1} cm^{-1} was used for DCIP at 600 nm at pH 8. The enzyme activity unit is nanomoles of dye reduced per second per milligram of protein.

Transhydrogenation Experiments. All experiments were carried out in 0.1 M phosphate buffer and 1 mM EDTA, pH 7.0, in the dark at 33 °C under argon, as described by Urban

[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; SDS, sodium dodecyl sulfate; $PheMeSO_2F$, phenylmethanesulfonyl fluoride.

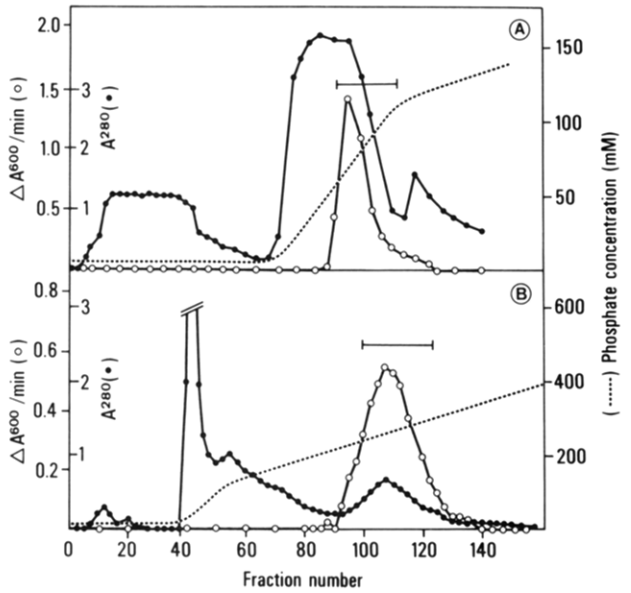


FIGURE 1: Purification of hydroxyacid oxidase by DEAE-cellulose chromatography (A) and hydroxyapatite chromatography (B). Experimental details are described under Methods. Fraction volumes were 6.8 mL for (A) and 6 mL for (B). The fractions pooled are indicated by horizontal bars.

Table I: Purification of L-2-Hydroxyacid Oxidase[a]

purifn step	total protein (mg)	activity (units mg^{-1}) total	activity (units mg^{-1}) specific	yield (%)	purifn factor
tissue homogenization	12920	1778	0.133	100	1
ammonium sulfate precipitation	2960	1938	0.65	109	5
DEAE-cellulose chromatography	352	1322	3.75	74	28
hydroxyapatite chromatography	31	653	21.1	37	158

[a] Based on 100 g of rat kidney.

et al. (1983). The total 2-keto acid concentration in the transhydrogenation mixtures was determined by using NADH and beef heart lactate dehydrogenase as described (Urban et al., 1983). The total 2-hydroxy acid concentration was titrated with flavocytochrome b_2 from baker's yeast in the presence of excess ferricyanide. Bromopyruvate was quantified with the 5-thio-2-nitrobenzoate dianion at pH 8.0 (Yun & Suelter, 1978).

Results

Purification of L-*2-Hydroxyacid Oxidase.* After tissue disruption, the enzyme was purified by a three-step procedure described under Methods: ammonium sulfate fractionation followed by chromatography on DEAE-cellulose and then on hydroxyapatite. Figure 1 shows the elution profiles for the two columns. After the first one, the pooled fractions showed a distinct flavin spectrum, but a small absorption band at 410 nm indicated that one of the contaminants was a hemoprotein, probably residual hemoglobin. The enzyme was eluted from hydroxyapatite at about 0.25 M phosphate; the pooled fractions appeared to be pure, as judged by SDS–polyacrylamide gel electrophoresis (Figure 2). Their specific activity at 30 °C was 20–25 nmol of DCIP reduced s^{-1} (mg of protein)$^{-1}$. A summary of the purification results is given in Table I. The enzyme could be stored for months at 4 °C after ammonium sulfate precipitation. We also found it stable to freezing in 0.1 M Tris-HCl buffer, pH 8.4.

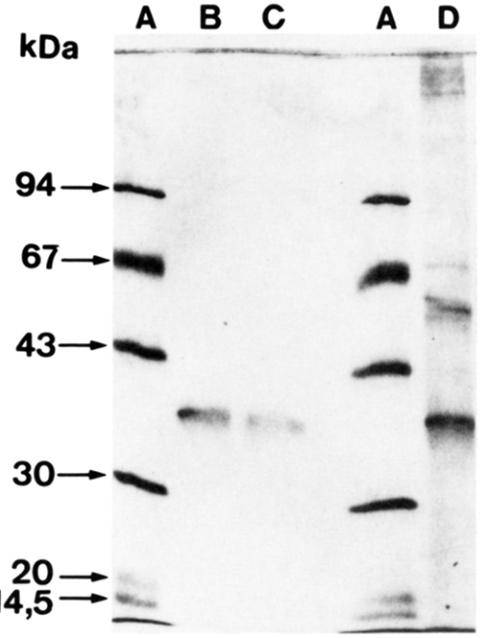


FIGURE 2: SDS–polyacrylamide gel electrophoresis of purified hydroxyacid oxidase. The gel (12% acrylamide) was stained with Coomassie blue. (Lane A) Molecular weight markers; (lanes B and C) hydroxyacid oxidase after hydroxyapatite chromatography (4 and 2 μg, respectively); (lane D) enzyme after DEAE-cellulose chromatography.

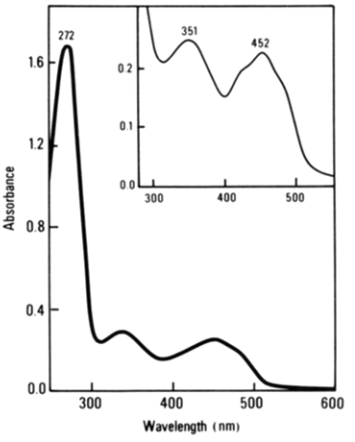


FIGURE 3: Absorption spectrum of purified hydroxyacid oxidase. The buffer was 0.1 M Tris-HCl, pH 8. Enzyme concentration was 19.4 μM.

Physicochemical Characterization of Purified Hydroxyacid Oxidase. An apparent M_r of 39 000 was determined for the enzyme on a calibrated SDS–polyacrylamide electrophoresis gel under reducing conditions. The absorbance of an enzyme solution at 1 mg/mL in 0.1 M Tris-HCl buffer, pH 8, was 1.88 (the protein concentration was evaluated by amino acid analysis).

The protein showed absorption maxima at 272, 351, and 452 nm, typical of a flavoprotein (Figure 3). As already observed by Cromartie and Walsh (1975a), the low-wavelength flavin band at 351 nm is blue-shifted relative to that in most other flavoenzymes (370–390 nm). Using an M_r of 39 000 and a protein estimate after amino acid analysis, we found a molar extinction coefficient at 452 nm of 11 700 M^{-1} cm^{-1} at pH 8.

These results implicitly assume the presence of one FMN per subunit. This point was verified by fluorometric titration of the cofactor after protein denaturation in 6 M Gdn-HCl, as described under Methods. The experiment yielded a

Table II: Amino Acid Composition of L-2-Hydroxyacid Oxidase[a]

amino acid	Duley and Holmes (1976)	this work
Asp	34	31.5 ± 2.0
Thr	18	14.6 ± 0.8
Ser	24	21.7 ± 1.7
Glu	38	35.5 ± 2.4
Pro	16	15.5 ± 1.1
Gly	25	24.1 ± 2.0
Ala	34	30.7 ± 1.8
Val	23	21.1 ± 0.8
Met	6	5.6 ± 1.0
Ile	ND	22.4 ± 1.3
Leu	36	36
Tyr	9	7.9 ± 0.5
Phe	14	13.3 ± 0.7
His	7	6.4 ± 0.7
Lys	24	23.7 ± 1.3
Arg	20	20.3 ± 1.6
Trp	5[b]	9.9 ± 0.9[c]
Cys	5[d]	8.9 ± 0.5[e]

[a] Determined as described under Methods. The presented data are the average of nine analyses. ND = not determined. The number of residues for each amino acid is calculated on the basis of 36 Leu per subunit, which gives an approximate M_r of 39 000. [b] Determined after hydrolysis with methanesulfonic acid (Lui & Chang, 1971) and by spectrophotometric titration (Beaven & Holiday, 1952). [c] Determined by spectrofluorometric titration as described under Methods. [d] Determined as half-cystine by amino acid analysis. [e] Determined as SCM-cysteine by amino acid analysis.

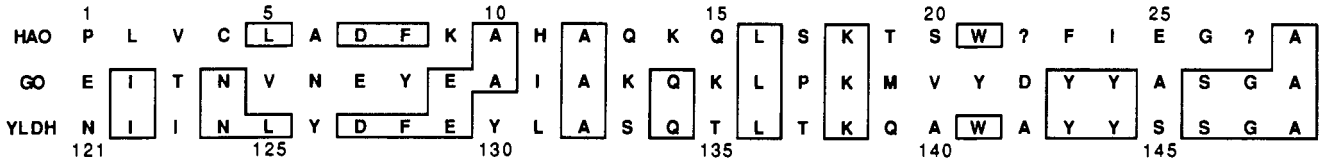
stoichiometry of 0.91 ± 0.11 FMN/39 kDa.

The amino acid composition of hydroxyacid oxidase is given in Table II, calculated on the basis of 36 leucine residues for a molecular weight of about 39 000. It is in satisfactory agreement with that published by Duley and Holmes (1976) except for half-cystine and tryptophan. The results of an Edman degradation of the protein are given in Table III, where the determined sequence is aligned with those of spinach glycolate oxidase (Volokita & Somerville, 1987) and of the flavodehydrogenase domain of baker's yeast flavocytochrome b_2 (Lederer et al., 1985; Guiard, 1985).

Specificity of Hydroxyacid Oxidase. The pH optimum for the enzyme was found to be 8.4 in 0.1 M phosphate buffer (Cromartie & Walsh, 1975a), 8.0 in 33 mM phosphate buffer (Nakano & Danowski, 1966), and between 8.6 and 8.9 in 33 mM Tris buffer (Domenech et al., 1973). K_m and V_{max} values for various substrates were determined by Cromartie and Walsh (1975a) and Domenech et al. (1973). For our projected experiments with bromopyruvate (see below) we needed kinetic data at pH 7, because of the instability of this compound at higher pH values. Therefore, we determined kinetic parameters for a number of substrates in phosphate buffer at pH 7. These are presented in Table IV, together with values obtained at pH 7.5 in imidazole buffer. We also found that phosphate ions may inhibit the enzyme, since in phosphate buffer at pH 7.5 the V_{max} for 2-hydroxybutyrate oxidation was 12.1 units mg^{-1} compared to 26.2 units mg^{-1} for the same substrate in imidazole buffer at the same pH. Generally speaking, our kinetic data are in fair agreement with those of Domenech et al. (1973). Our K_m values, however, differ from those published by Cromartie and Walsh (1975a) more than would be expected from the difference in assay conditions.

Reversibility of Hydroxy Acid Dehydrogenation and Bromide Ion Elimination. We incubated hydroxyacid oxidase under argon in the presence of saturating L-mandelate concentrations and various concentrations of bromopyruvate, as had been done with flavocytochrome b_2, lactate, and bromopyruvate (Urban et al., 1983). Initial rates could be easily measured over the first 10–15 min of incubation. Figure 4 shows that bromopyruvate disappearance is enzyme-catalyzed, since it is a saturable phenomenon. From figure 4, the following values can be derived: K_m = 4.4 ± 0.5 mM and k_{cat} = 0.36 ± 0.04 s^{-1}. In the presence of hydroxyacid oxidase without mandelate or of mandelate and enzyme inactivated

Table III: N-Terminal Sequence of Hydroxyacid Oxidase[a]



[a] Degradation was carried out as described under Methods. Two experiments were carried out. The first one with 160 pmol of S-carboxymethylated protein yielded the identity of 23 residues with an initial coupling yield of 32% and an average repetitive yield of 93.8%. The results of the second experiment are shown below. It was carried out with 1 nmol of unmodified protein with an initial coupling yield of 5% and a repetitive yield of 94.9%. The protein sequence of baker's yeast flavocytochrome b_2 [L-lactate dehydrogenase (YLDH)] is taken from Lederer et al. (1985); that of spinach glycolate oxidase (GO) is deduced from a cDNA sequence (Volokita & Somerville, 1987). Residue 1 of hydroxyacid oxidase (HAO) is aligned with residue 2 of the deduced glycolate oxidase sequence, which is preceded by a methionine.

Table IV: Enzymatic Parameters for the Oxidation by L-2-Hydroxyacid Oxidase of Various 2-Hydroxy and 2-Amino Acids at 30 °C[a]

	in 0.1 M phosphate buffer, pH 7.0		in 0.1 M imidazole buffer, pH 7.5	
substrates	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
amino acids				
L-leucine	ND	ND	6 ± 2	0.5 ± 0.1
DL-methionine	ND	ND	4 ± 1	0.15 ± 0.05
L-tryptophan	ND	ND	35 ± 10	0.8 ± 0.3
hydroxy acids				
L-lactate	1.8 ± 0.5	5.8 ± 0.3	3.4 ± 0.5	8 ± 2
DL-3-chlorolactate	0.8 ± 0.1	23.8 ± 0.8	0.70 ± 0.02	40 ± 4
D,L-2-hydroxybutyrate	0.6 ± 0.1	12 ± 2	1.0 ± 0.1	26.2 ± 0.9
D,L-2-hydroxyvalerate	0.35 ± 0.02	8.5 ± 0.3	0.25 ± 0.03	24 ± 3
D,L-2-hydroxycaproate	0.25 ± 0.01	5.8 ± 0.4	0.15 ± 0.01	23 ± 1
L-2-hydroxyisocaproate	0.90 ± 0.05	12.7 ± 0.9	0.7 ± 0.3	36 ± 4
D,L-phenyllactate	0.10 ± 0.03	3.3 ± 0.1	0.10 ± 0.02	14.2 ± 0.9
L-mandelate	0.8 ± 0.1	21 ± 2	0.40 ± 0.02	39 ± 2

[a] For racemic substrates, the K_m is calculated relative to the L-hydroxy acid in the mixture. ND = not determined.

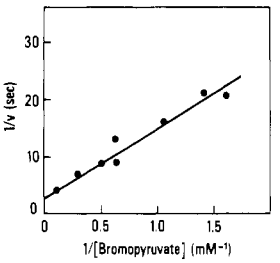


FIGURE 4: Concentration dependence of bromopyruvate disappearance. The reactions were carried out at 33 °C under argon, as described under Methods. At various time intervals, aliquots were withdrawn and immediately assayed for bromopyruvate content. Each point is the slope of a plot of bromopyruvate concentration versus time. Enzyme and L-mandelate concentrations were 8 μM and 20 mM, respectively.

Table V: Stoichiometries before and after Incubation with L-2-Hydroxyacid Oxidase[a]

time (min)	bromopyruvate (μmol)	total 2-keto acids (μmol)	total 2-hydroxy acids (μmol)
0	9.4	9.4	30.4
30	6.0	10.8	29.0

[a] The incubation was carried out under argon at 33 °C in 1 mL of 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA, 10 μM enzyme, 30.4 mM D,L-2-hydroxybutyrate, and 9.4 mM bromopyruvate. After 30 min, the reaction was quenched with HCl, and the concentrations were determined as described under Methods.

by hydroxybutynoate (Cromartie & Walsh, 1975b), bromopyruvate was stable.

The observed bromopyruvate disappearance could be due to a simple transhydrogenation reaction, but also, totally or partially, to an enzyme-catalyzed elimination reaction, as was shown for flavocytochrome b_2 (Urban et al., 1983) and for lactate oxidase from *Mycobacterium smegmatis* [Urban, unpublished experiments quoted in Lederer (1984)].

In the former case, total hydroxy acid and total keto acid concentrations must remain stable. But when an elimination reaction takes place, there will be net hydroxy acid disappearance and net keto acid production, since the balance is given by the two following turnover reactions:

$$\text{bromopyruvate} + \text{E, Fl}_{red} \rightarrow \text{pyruvate} + \text{Br}^- + \text{H}^+ + \text{E, Fl}_{ox}$$

$$\text{hydroxy acid} + \text{E, Fl}_{ox} \rightarrow \text{keto acid} + \text{E, Fl}_{red}$$

Table V shows the results of a transhydrogenation experiment between hydroxybutyrate and bromopyruvate. There was a decrease in total hydroxy acids and a corresponding increase in total keto acids. Since an excess of 1.4 μmol of keto acid appeared but 3.4 μmol of bromopyruvate disappeared, it can be concluded that 2 μmol of bromolactate were also formed. An experiment where [2-^{14}C]bromopyruvate was used showed that indeed both [2-^{14}C]bromolactate and [2-^{14}C]pyruvate were formed (in an approximate ratio of 1 to 10 after 90 min at 30 °C, starting with 21 μM enzyme, 30 mM L-lactate, and 20 mM [2-^{14}C]bromopyruvate).

DISCUSSION

Hydroxyacid Oxidase Purification and Characterization. The previously described purification methods for hydroxyacid oxidase from rat kidney (Nakano & Danowski, 1966; Cromartie & Walsh, 1975a; Duley & Holmes, 1976) used fractionations with ammonium sulfate or acetone and two chromatographic steps on DEAE-cellulose. Our scheme involves ammonium sulfate precipitation, only one DEAE-cellulose column, and chromatography on hydroxyapatite (Table I). The enzyme is obtained with a final yield of 35–40%. In Table VI are compared a number of parameters relative to the purified enzyme as determined by the various groups. Our results are in best agreement with those published by Duley and Holmes (1976). The most important discrepancy between the various authors concerns the flavin stoichiometry. A value of 0.5 per subunit was found only by Cromartie and Walsh (1975a) and may have been due to flavin loss during storage (Duley & Holmes, 1976). Since two laboratories obtained evidence for a tetrameric hydroxyacid oxidase (Cromartie & Walsh, 1975a; Phillips & al., 1976), we did not investigate this aspect of the structure at all.

A point worth noting is the low specific activity of the enzyme with all substrates tested, even though our preparation appears to be somewhat more active than those described previously. The figure of 21 units s^{-1} mg^{-1} we obtained for the V_{max} for mandelate (Table IV) corresponds to 0.86 mols of substrate converted s^{-1} (mol of enzyme)$^{-1}$. This suggests that indeed the true physiological substrate for L-hydroxyacid oxidase remains to be discovered, since the thiol–glyoxylate adducts tested by Brush and Hamilton (1981) were not processed at a higher rate than hydroxybutyrate.

An interesting point that emerges from our study is the sequence similarity between the N-terminus of hydroxyacid oxidase and two other FMN-linked enzymes: spinach glycolate oxidase and flavocytochrome b_2 from baker's yeast. It can be surmised that the spinach enzyme is the plant counterpart of mammalian glycolate oxidase or short-chain hydroxyacid oxidase. Duley and Holmes (1976) compared a number of properties of the rat kidney long-chain oxidase and rat liver short-chain oxidase, which they called isozymes B and A, respectively. On the basis of similarity in molecular weights, amino acid composition, and immunological cross-reactivity, they proposed that the two enzymes were the products of recently duplicated genes. Our sequencing results lend support to this suggestion. Of 26 positions compared between rat kidney hydroxyacid oxidase and spinach glycolate oxidase (Volokita & Somerville, 1987), one finds 5 identical residues and 13 positions differing by one base change in their codons.

The similarity to positions 121–149 of flavocytochrome b_2 (Lederer et al., 1985; Guiard, 1985) is even stronger: 8 identities and 9 single base changes. A complete sequence comparison between spinach glycolate oxidase and the flavo-

Table VI: Characterization of L-2-Hydroxyacid Oxidase

parameters	Nakano and Danowski (1966)	Cromartie and Walsh (1975a)	Duley and Holmes (1976)	this work
subunit mol wt	49 300	47 500	40 000	39 000
FMN stoichiometry (mol/subunit)	1.0	0.5	1.0	0.9
absorption coefficients				
ϵ^{M}_{450} (M^{-1} cm^{-1})	12 700	ND[a]	11 500	11 700
$\epsilon^{mg/mL}_{280}$	1.7	0.9	1.9	1.9
A_{280}/A_{450} ratio	6.5	6.8–5.2	4.7	6.3–5.2
specific activity (nmol s^{-1} mg^{-1})	11.8[b]	8[c]	14.7[d]	20[e]

[a] ND = not determined. [b] At pH 7.9 and 37 °C. [c] At pH 8.4 and 25 °C. [d] At pH 8.0 and 30 °C. [e] At pH 8.4 and 30 °C.

dehydrogenase domain of flavocytochrome b_2 shows about 40% identity. Furthermore, both proteins present a flavodehydrogenase domain folded as a $\beta_8\alpha_8$-barrel (Xia et al., 1987; Lindquist & Brändén, 1985). The FMN is bound to the two structures in a very similar fashion, and the root mean square deviation between the position of about 325 equivalent C_α atoms was found to be about 1.3 Å (Mathews & Xia, 1987). This figure is much smaller than that obtained in the comparison of any member of the pair with other $\beta_8\alpha_8$-barrel enzymes devoid of flavin. These results show that flavocytochrome b_2 and glycolate oxidase are homologous. The sequence segments compared here correspond to an N-terminal extension before entering the β-barrel, an extension that presents several short helical segments. The limited sequence data presented here strongly suggest that long-chain hydroxyacid oxidase belongs to the same evolutionary family. It will be interesting to elucidate the critical structural features that have made one gene product capable of reacting with oxygen and the other of reacting with monoelectronic acceptors.

More Evidence in Favor of a Carbanion Mechanism for Hydroxyacid Oxidase. Three main pieces of evidence have been adduced in favor of carbanion formation as the first step in flavoprotein-catalyzed dehydrogenation reactions of α-hydroxy and α-amino acids. One piece of evidence is the reaction between nitroethane and D-amino-acid oxidase (Porter et al., 1973). Another piece of evidence, for the hydroxy acid oxidizing enzymes, is suicide inactivation by 2-hydroxy-3-butynoate. This reagent inactivated all enzymes tested thus far: L-lactate oxidase from *M. smegmatis* (Ghisla et al., 1976; Schonbrunn et al., 1976), D- and L-lactate dehydrogenases from *Escherichia coli* (Walsh et al., 1972), flavocytochrome b_2 from baker's yeast (Lederer, 1974; Pompon & Lederer, 1985), D-lactate dehydrogenase from *Maegasphaera elsdenii* (Ghisla et al., 1979; Olson et al., 1979), pea glycolate oxidase (Fendrich & Ghisla, 1982), and rat kidney hydroxyacid oxidase (Cromartie & Walsh, 1975b). The third piece of evidence in favor of carbanion formation is the dehydrohalogenation of β-halogenoamino acids and β-halogenohydroxy acids (Walsh et al., 1971, 1973a,b; Voet et al., 1972; Miyake et al., 1973; Bright & Porter, 1975). However, a few flavoenzymes proved incapable of carrying out this reaction under normal turnover conditions: Hydroxyacid oxidase and flavocytochrome b_2 were in that case, as mentioned above (Lederer, 1974; Cromartie & Walsh, 1975b). The rationale put forth at the time was that the putative carbanion was formed slowly but yielded its electrons too rapidly to flavin for elimination to have a chance to take place.

This hypothesis has been recently verified for flavocytochrome b_2. We have shown that under transhydrogenation conditions, starting with reduced enzyme and a halogenopyruvate, it is possible to observe halide ion elimination because the carbanion, once formed at the expense of reduced flavin, is protonated slowly (Urban & Lederer, 1984, 1985). Indeed, the partition ratio between carbanion oxidation and halide ion elimination was about 500, while the partition ratio between carbanion protonation and elimination was about 2 (Urban & Lederer, 1984). Therefore, under conditions where the carbanion is forced to the hydroxy acid (reverse reaction), elimination is experimentally easier to observe.

In this paper, we show that under conditions similar to those used for flavocytochrome b_2 hydroxyacid oxidase is also capable of forming pyruvate from bromopyruvate in a reductive elimination process. The present study is not as detailed as that carried out with flavocytochrome b_2. However, when combined with other available data, like deuterium isotope effects (Lederer, 1974; Urban & Lederer, 1985; Cromartie & Walsh, 1975b), it explains by analogy why it was impossible to obtain evidence for the dehydrohalogenation reaction in the presence of oxygen: the partition ratio was lying too much in favor of carbanion oxidation. With enzymes like D-amino-acid oxidase and lactate oxidase, for which product release constitutes the rate-limiting step (Lockridge et al., 1972; Bright & Porter, 1975), the Michaelis complex E_{red}–halogenoketo acid has time to return partially to the complex E_{OX}–carbanion, so that elimination can be observed under the conditions of the forward reaction. Therefore, it is the difference in the rate-limiting step of the overall reaction that determines the difference in behavior of a number of flavoproteins toward β-halogeno substrates in spite of a common chemical mechanism.

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REFERENCES

Angermüller, S., Leupold, C., Zaar, K., & Fahimi, H. D. (1986a) *Histochemistry 85*, 411–418.

Angermüller, S., Leupold, C., Völkl, A., & Fahimi, H. D. (1986b) *Histochemistry 85*, 403–409.

Beaven, G. H., & Holiday, E. R. (1952) *Adv. Protein Chem. 2*, 319–350.

Blanchard, M., Green, D. E., Nocito, V., & Ratner, S. (1945) *J. Biol. Chem. 161*, 583–598.

Blanchard, M., Green, D. E., Nocito-Carroll, V., & Ratner, S. (1946) *J. Biol. Chem. 163*, 137–144.

Bright, H. J., & Porter, D. J. T. (1975) *Enzymes (3rd Ed.) 12*, 421–505.

Brush, E. J., & Hamilton, G. A. (1981) *Biochem. Biophys. Res. Commun. 103*, 1194–1200.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Cromartie, T. H., & Walsh, C. T. (1975a) *Biochemistry 14*, 2588–2596.

Cromartie, T. H., & Walsh, C. T. (1975b) *Biochemistry 14*, 3482–3490.

Domenech, C. E., Machado de Domenech, E. E., & Blanco, A. (1973) *Biochim. Biophys. Acta 321*, 54–63.

Douglas, M., Finkelstein, D., & Butow, R. A. (1979) *Methods Enzymol. 56*, 58–66.

Duley, J. A., & Holmes, R. S. (1976) *Eur. J. Biochem. 63*, 163–173.

Fendrich, G., & Ghisla, S. (1982) *Biochim. Biophys. Acta 202*, 242–248.

Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H., & Walsh, C. T. (1976) *Biochemistry 15*, 1791–1797.

Ghisla, S., Olson, S. T., Massey, V., & Lhoste, J. M. (1979) *Biochemistry 18*, 4733–4742.

Guiard, B. (1985) *EMBO J. 4*, 3265–3272.

Lederer, F. (1974) *Eur. J. Biochem. 46*, 393–399.

Lederer, F. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 513–526, de Gruyter, Berlin.

Lederer, F., Ghrir, R., Guiard, B., Cortial, S., & Ito, A. (1983) *Eur. J. Biochem. 132*, 95–102.

Lederer, F., Cortial, S., Becam, A. M., Haumont, P. Y., & Perez, L. (1985) *Eur. J. Biochem. 152*, 419–428.

Lindquist, Y., & Brändén, C. I. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6855–6859.
Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097–8106.
Lui, T. Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842–2848.
Mathews, F. S., & Xia, Z. X. (1987) in *Flavins and Flavoproteins* (McCormick, D. B., & Edmondson, D. E., Eds.) pp 123–132, de Gruyter, Berlin.
McGroarty, E., Hsieh, B., Wied, D. M., Gee, R., & Tolbert, N. E. (1974) *Arch. Biochem. Biophys. 161*, 194–210.
Miyake, T., Abe, T., & Yamano, T. (1973) *J. Biochem. (Tokyo) 73*, 1–11.
Nakano, N., & Danovski, T. S. (1966) *J. Biol. Chem. 241*, 2075–2083.
Olson, S. T., Massey, V., Ghisla, S., & Whitfield, C. D. (1979) *Biochemistry 18*, 4724–4732.
Pajot, P. (1976) *Eur. J. Biochem. 63*, 263–269.
Phillips, D. R., Duley J. A., Fennell, D. J., & Holmes, R. S. (1976) *Biochim. Biophys. Acta 427*, 679–687.
Pompon, D., & Lederer, F. (1985) *Eur. J. Biochem. 148*, 145–154.
Porter, D. J. T., Voet, J. G., & Bright, H. J. (1973) *J. Biol. Chem. 248*, 4400–4416.
Robinson, J. C., Keay, L., Molinari, R., & Sizer, I. W. (1962) *J. Biol. Chem. 237*, 2001–2010.
Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H., & Massey, V. (1976) *Biochemistry 15*, 1798–1807.
Tolbert, N. E. (1981) *Annu. Rev. Biochem. 50*, 133–157.
Urban, P., & Lederer, F. (1984) *Eur. J. Biochem. 144*, 345–351.
Urban, P., & Lederer, F. (1985) *J. Biol. Chem. 260*, 11115–11122.
Urban, P., Alliel, P. M., & Lederer, F. (1983) *Eur. J. Biochem. 134*, 275–281.
Urban, P., Chirat, I., & Lederer, F. (1987) *Abstr. 18th FEBS Meet.*, No. Tu S-15.
Voet, J. G., Porter, D. J. T., & Bright, H. J. (1972) *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 27B*, 1054–1055.
Volokita, M., & Somerville, C. R. (1987) *J. Biol. Chem. 262*, 15825–15828.
Walsh, C. T., Shonbrunn, A., & Abeles, R. H. (1971) *J. Biol. Chem. 246*, 6855–6866.
Walsh, C., Abeles, R., & Kaback, H. (1972) *J. Biol. Chem. 247*, 7858–7863.
Walsh, C. T., Krodel, E., Massey, V., & Abeles, R. H. (1973a) *J. Biol. Chem. 248*, 1946–1955.
Walsh, C. T., Lockridge, O., Massey, V., & Abeles, R. H. (1973b) *J. Biol. Chem. 248*, 7049–7054.
Xia, Z. X., Shamala, N., Bethge, P. H., Lim, L. W., Bellamy, H. D., Xuong, N. H., Lederer, F., & Mathews, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2629–2633.
Yun, S. L., & Suelter, C. H. (1978) *Anal. Biochem. 85*, 437–441.

Equilibrium of 5,6-Hydration of NADH and Mechanism of ATP-Dependent Dehydration†

Scott A. Acheson,‡ Henry N. Kirkman,§ and Richard Wolfenden*,‡
Departments of Biochemistry and Pediatrics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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ABSTRACT: At equilibrium, water addition to the 5,6 double bond of NADH was observed to favor the hydrate by a factor of ~100. Hydration generates two epimers of NADHX (β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide). Only the 6*S* epimer of the hydrate was found to serve as a true substrate for an ATP-dependent dehydratase from yeast that regenerates NADH. Yet enzymatic conversion of both epimers of the hydrate to NADH was found to proceed essentially to completion in the presence of ATP and dehydratase. This is explained by the observed ability of the epimers to undergo rapid spontaneous equilibration, so that it is unnecessary to postulate a lack of stereospecificity in the dehydratase.

Glyceraldehyde 3-phosphate dehydrogenase (GPDH)[1] catalyzes the conversion of NADH to a hydrated compound that is inactive in the reactions normally catalyzed by this dehydrogenase (Rafter et al., 1954; Chaykin et al., 1956). Hydration of NADH also proceeds spontaneously at a slower rate under neutral or mildly acidic conditions, and the product is β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide (NADHX) (Scheme I). Oppenheimer and Kaplan (1974b) established by ^{1}H NMR that the product consists of a mixture of C-6 epimers: about 60% *S* and 40% *R*.[2] These epimers can be separated by HPLC (Miksic & Brown, 1978; Margolis et al., 1978).

Hydration of NADH would presumably tend to result in depletion of this coenzyme, and the 5,6-hydrate of NADPH has been found to act as a strong inhibitor of glucose 6-

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* Author to whom correspondence should be addressed.
‡ Department of Biochemistry.
§ Department of Pediatrics and Biological Sciences Research Center.

[1] Abbreviations: NADHX, β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide; c(THN)AD, cyclotetrahydronicotinamide adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride; GPDH, glyceraldehyde 3-phosphate dehydrogenase.

[2] The *S* epimer corresponds to hydroxylation on the "A" face of NADH, and the *R* epimer corresponds to hydroxylation on the "B" face.

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