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Hydroxamates as Substrates and Inhibitors for FMN-Dependent 2-Hydroxy Acid Dehydrogenases[1]

David Amar,* Paul North,* Vanda Miskiniene,† Narimantas Cénas,†
and Florence Lederer*,[2]

**Laboratoire d'Enzymologie et de Biochimie Structurales, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France; and †Biochemijos Institutas,*
Mokslininku 12, 2600 Vilnius, Lithuania

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Long-chain hydroxy acid oxydase (HAO) is a member of a flavoenzyme family with significant amino acid sequence similarity and strongly conserved three-dimensional structure; in particular, active-site amino acids involved in catalysis are invariant, with one exception, and numerous enzymatic studies suggest an identical chemical mechanism involving an intermediate carbanion for all family members. Known physiological substrates are a variety of L-2-hydroxy acids. Peroxisomal HAO differs from the other family members in that its actual physiological substrate is not known; it was first described as an L-amino acid oxidase, and recently was identified as an enzyme that converts creatol (hydroxycreatinine) to methylguanidine (a metabolite involved in a variety of uremic syndromes). Creatol (2-amino-5-hydroxy-1-methyl-4(5H)imidazolone) is not a 2-hydroxy acid. We show in this work that 2-hydroxyphenyl acetohydroxamate (HYPAH, the hydroxamate of mandelic acid), a compound that bears similarity both to mandelate (one of the best substrates known) and to creatol, is turned over by HAO, but between 10- and 100-fold less efficiently than mandelate itself. The compound also binds to the active site of homologous flavocytochrome b_2 (L-lactate dehydrogenase). Comparative pH-rate studies for mandelate and its hydroxamate suggest that HYPAH may bind in its ionized form. Both pH-rate profiles are bell-shaped curves, as are those determined for two other family members, flavocytochrome b_2 and mandelate dehydrogenase; while the group with an acid pK_a between 5 and 6 is most likely the active-site histidine (the residue which abstracts the substrate C2 proton), the identity of the basic group is less clear. It has been proposed to be one of the active site arginines (Lehoux, I., and Mitra, B. (1999) *Biochemistry* **38,** 5836–5848); we suggest as an alternative that it could be the lysine residue that interacts with the flavin N1 and O2 positions and stabilizes the negative charge of reduced flavin. In addition to these studies, we have found that HAO is competitively inhibited by benzohydroxamate, which is one atom shorter than HYPAH; its affinity is nearly 100-fold lower than that of the substrate, in contrast to the strong inhibition it exerts on mandelate racemase (Maurice, St. M., and Bearne, S. L. (2000) *Biochemistry* **39,** 13324–13335). In the latter case, the 100-fold higher affinity compared

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[1] This paper is dedicated to Professor Frank H. Westheimer on the occasion of his 90th birthday.

[2] To whom correspondence and reprint requests should be addressed. Fax: 33 1 69 82 31 29. E-mail: lederer@lebs.cnrs-gif.fr.

[3] Abbreviations used: DCIP, dichlorophenol indophenol; FDH, Flavodehydrogenase domain of flavocytochrome b_2; Flb2, flavocytochrome b_2 (L-lactate dehydrogenase) from *Saccharomyces cerevisiae*; GOX, glycolate oxidase from spinach; LMO, long-chain L-2-hydroxy acid oxidase from rat kidney; HYPAH, L-2-hydroxy-phenyl acetohydroxamic acid; LMO, L-lactate 2-monooxygenase from *Mycobacterium smegmatis*; MDH, L-mandelate dehydrogenase from *Pseudomonas putida*; WT, wild-type.

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to mandelate was proposed to arise from the fact that the hydroxamate can mimic the enolic intermediate which lies on the reaction pathway after C2 proton abstraction. Thus our results do not support the existence of a similar enolic intermediate for HAO (and probably its homologues), although they do not disprove it.

Long chain hydroxy acid oxidase (HAO) (EC 1.1.3.15) from rat kidney is a tetrameric peroxisomal flavoenzyme, which belongs to the family of FMN-dependent 2-hydroxy acid-oxidizing enzymes. It is an isozyme (isozyme B) of its closest homologue glycolate oxidase (GOX) or short chain hydroxy acid oxidase from plants and animals (EC 1.1.3.15, isozyme A) (*1*). Other well-studied family members are flavocytochrome b_2 or L-lactate dehydrogenase (Flb2) (*2*), lactate monooxygenase (LMO) from *Mycobacterium smegmatis* (*3*), lactate oxidase from *Aerococcus viridans* (*4*), and mandelate dehydrogenase from *Pseudomonas putida* (MDH) (*5*). The family comprises two subclasses, the dehydrogenases-oxidases for which the flavin oxidative half-reaction requires oxygen, and the dehydrogenases-electron transferases for which the oxidative half-reaction requires a monoelectronic acceptor. Flb2 contains a built-in acceptor, the heme-binding domain.

Refined crystal structures have been published for GOX (*6*), Flb2 (*7*), and more recently for a soluble form of MDH (*8*); that of HAO is being refined (*9*). A comparison of structural data shows an identical $\beta_8\alpha_8$ barrel, and invariant catalytic residues in the active sites (*10*) (Fig. 1). These residues are also conserved in homologous

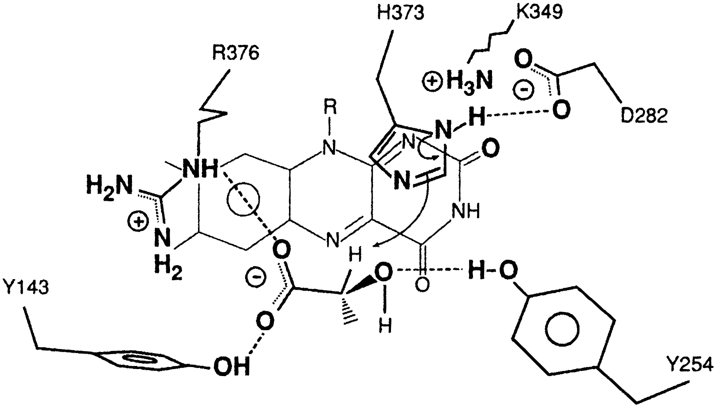
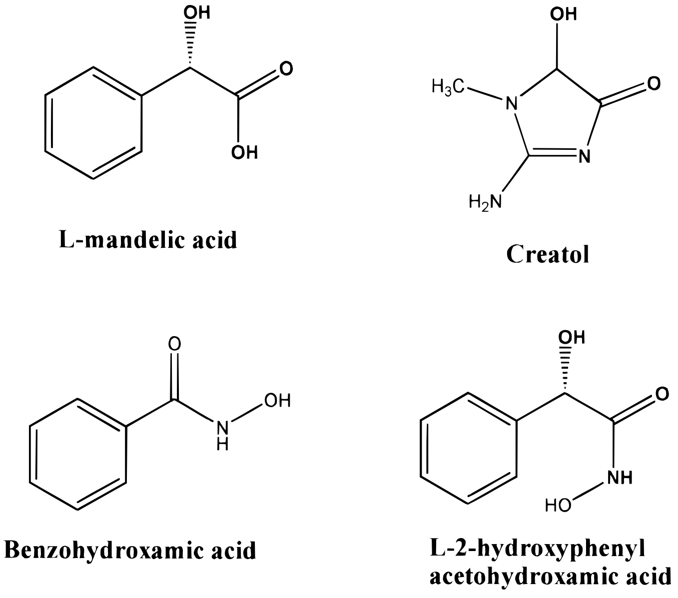


FIG. 1. The flavocytochrome b_2 active site. The representation is taken from the structure determined by Xia and Mathews (*7*) (1FCB). The positioning of lactate is adapted from that of pyruvate observed in the crystal structure (*15*). Arg289 is not pictured here; it lies close to Arg376, above the plane of the figure, and in the unliganded enzyme its guanido group is turned away from the active site and forms an ion pair with invariant Asp292 (*7*); crystallographic evidence, confirmed by site-directed mutagenesis, indicates it can move toward the substrate and interact with it (*8, 44–47,53*). The numbering of equivalent residues is for Flb2: Tyr143 Tyr254 Asp282 Arg289 Asp292 Lys349 His373 Arg376; for HAO: Phe23 Tyr129 Asp157 Arg164 Asp167 Lys223 His247 Arg250; for GOX: Tyr24 Tyr129 Asp157 Arg164 Asp167 Lys230 His254 Arg257; for MDH: Tyr26 Tyr131 Asp158 Arg165 Asp168 Lys250 His274 Arg277. The arrows depict abstraction of the substrate α-proton by His373.

sequences found in data banks, except for Tyr143 (Flb2), which in HAO is Phe23. Recently, the genes of three human homologues were cloned and expressed, one of which is closer in sequence to GOX and has Tyr at the corresponding position, and the two others have a Phe instead and are more similar to HAO (*11*). In any case, the structural data, combined with numerous mechanistic studies suggest an identical chemical mechanism in the family for the flavin reductive half-reaction, in other words for substrate dehydrogenation (*12*). The reaction is generally thought to proceed via a carbanion mechanism, with His373 (Flb2) and its homologues abstracting the substrate C2 proton, thus generating a carbanion (*12,13*) (Fig. 1). The latter could either form a transient covalent adduct with the flavin and then decompose to reduced flavin and keto acid, or yield the reducing equivalents to the flavin in a quick succession of two monoelectronic transfers (*12,14,15*).

The physiological substrate of HAO is not known. The enzyme was first described as an L-amino acid oxidase (*16*), but was then found more active with L-2-hydroxy acids, especially those with a long chain (*17*). Over the years, HAO was also proposed to act on adducts between glyoxylate and sulfhydryl compounds (*18,19*), to be capable of oxidizing thyroxine and triiodothyronine (*20*), to contribute to the metabolism of some nephrotoxic cysteine conjugates such as 1,2-dichlorovinylcysteine (*21,22*). Recently, the human HAO homologues were suggested to participate in fatty acid α-oxidation (*11*). More surprisingly, the enzyme responsible for the production of methylguanidine (a metabolite involved in a variety of uremic syndromes) from creatol was identified as HAO after purification and gene cloning (*23,24*). This was unexpected because creatol (hydroxycreatinine, or 2-amino-5-hydroxy-1-methyl-4(5H)imidazolone) is not a 2-hydroxy acid (Scheme 1).



L-mandelic acid

Creatol

Benzohydroxamic acid

L-2-hydroxyphenyl acetohydroxamic acid

SCHEME 1.

In this work, we show that replacing the carboxylate function of mandelate, one of the best HAO substrates, with a hydroxamate function, which shows partial similarity to creatol (Scheme 1) yields a compound which is an HAO substrate and can also bind to Flb2. These results led us to examine the pH dependence of HAO activity both with mandelate and its hydroxamate, in order to find out if the latter binds in the neutral or the anionic form. Furthermore, in view of the use of certain hydroxamates as transition state analogues (for example phosphoglycolohydroxamate (*25*), phosphonoacetohydroxamate (*26*), benzohydroxamate (*27*)), we also analyzed the inhibition of HAO by benzohydroxamic acid (Scheme 1).

MATERIALS AND METHODS

Materials. L-Mandelic acid, benzoylformic acid, and benzohydroxamic acid were purchased from Sigma-Aldrich. Recombinant HAO (isozyme $\beta 1$) was purified as described (*28*) with the following modification: the second chromatographic step was carried out on DEAE Sepharose Fast Flow (Pharmacia), which gave a better yield for this step than DEAE cellulose. The enzyme concentration was estimated using an ε_M of 11 700 M^{-1} cm^{-1} at 453 nm (*29*). The recombinant flavodehydrogenase domain (FDH) of flavocytochrome b_2 was purified from *E. coli* as described, except that deoxyribonuclease and ribonuclease were omitted (*30*).

Synthesis of L*-2-hydroxy-2-phenyl acetohydroxamic acid (HYPAH).* The synthesis was carried out in two steps according to (*31*), with an overall yield of 40%. ^1H NMR (DMSO): 10.73 (*s*, N-OH), 8.78 (*s*, NH), 7.42 (*m*, 2 arom. ortho H), 7.3 (*m*, 3 arom. meta, para H), 5.95 (*d*, OH), 4.89 (*d*, H-C(2)). M.p. 126.1°C. $[\alpha]_D^{25} = -56°$ (c = 1 mg/ml, in CH_3OH). The ^1H NMR data were collected on a 300 MHz Brücker AC300 instrument.

Enzymatic assays. They were carried out with a Uvikon 943 spectrophotometer. For HAO, steady-state assays were routinely performed in the standard assay solution consisting of 70 μM DCIP in 0.1 M imidazolium/HCl buffer, 1 mM EDTA, pH 7.5, at 30°C with 0.6 μM enzyme. At this pH, the ε_M for DCIP is 21,000 M^{-1} cm^{-1} at 600 nm. Data were fitted to the Michaelis–Menten equation (Eq. [1]) using a least-squares iterative nonlinear program (Kaleidagraph 3.5). For inhibition studies, kinetics were performed under the same conditions, using L-mandelate as a substrate. The Dixon representation was used to determine kinetic parameters. The data were fitted to Eq. [2] for competitive inhibition (*32*) and to Eq. [3] for mixed (competitive, K_i, and uncompetitive, K_{iu}) inhibition (*33*) using a least-squares iterative linear regression program. The enzyme activity is expressed in mol dye reduced.s^{-1} = mol substrate oxidized.s^{-1}

For the FDH, inhibition studies were carried out using 13 mM potassium ferricyanide in 0.1 M phosphate buffer, 1 mM EDTA, pH 7, at 30°C in 2 mm cuvettes. L-Lactate was used as substrate.

$$v = \frac{V_{\max}[S]}{K_M + [S]} \qquad [1]$$

$$1/v = \frac{K_M(1 + [I]/K_i) + [S]}{V_{\max}[S]} \qquad [2]$$

$$[S]/v = \frac{1}{V_{\max}}(1 + [I]/K_{ic})\left(\frac{K_M(1 + [I]/K_{ic})}{1 + [I]/K_{iu}} + [S]\right) \qquad [3]$$

pH-dependence studies. For HAO, rates were again measured by monitoring the reduction of 70 μM DCIP at 600 nm. The following universal buffer was used over the pH range 5–9.6: 0.025 M boric acid, 0.025 M potassium phosphate, 0.025 M Tris and 0.025 M succinic acid; the pH was adjusted with KOH, and the ionic strength was adjusted to $I = 0.04$ with KCl. As the DCIP spectrum is not constant over the whole pH range, we used the molar absorptivity coefficients given by Armstrong (*34*). The data were first fitted to Eq. [1] to obtain k_{cat} and K_{M} values. They were then fitted, for k_{cat} and $k_{\mathrm{cat}}/K_{\mathrm{M}}$, to a bell-shaped curve using Eq. [4], where y is the experimental value at each pH and Y is the limiting value at intermediate pH

$$y = \frac{Y}{1 + 10^{pK_1 - pH} + 10^{pH - pK_2}} \qquad [4]$$

RESULTS

Steady-State Kinetics with HAO and the FDH

Although this has never been tested rigorously, HAO probably follows ping-pong kinetics, as is the case for glycolate oxidase (*35*), mandelate dehydrogenase (*5*) and the flavodehydrogenase domain (FDH) of Flb2 (N. Cénas, unpublished experiments). In this work, kinetic parameters were all determined at the constant DCIP concentration of 70 μM which was used in all previous work (*28,29,36*). We actually found that DCIP acts as an inhibitor at high concentrations, and determined for this mediator K_{M} and K_{i} values of 22 μM and 167 μM, respectively, with an optimum rate between 50 and 70 μM.

Mandelate hydroxamate (HYPAH) turned out to be a substrate for HAO. Its kinetic parameters are compared in Table 1 to those already determined under the same conditions for mandelate, phenyllactate, and lactate. At pH 7.5, in imidazolium buffer, HAO is about 100-fold more efficient with mandelate than with its hydroxamate,

TABLE 1

Steady-State Kinetic Parameters for Mandelate and Its Hydroxamate

Substrates	K_{M} (mM)	k_{cat} (s^{-1})	$k_{\mathrm{cat}}/K_{\mathrm{M}}$ ($\mathrm{mM}^{-1}\ \mathrm{s}^{-1}$)
L-Mandelic acid	0.17 ± 0.01	1.53 ± 0.02	9.0 ± 0.6
HYPAH	11.4 ± 1.5	1.20 ± 0.05	0.10 ± 0.01
L-Phenyllactic acid[a]	0.09 ± 0.02	0.59 ± 0.04	6.6 ± 1.9
L-Lactic acid[a]	6.1 ± 1.7	0.26 ± 0.02	0.04 ± 0.01

Note. Experimental data were obtained at 30°C in 0.1 M imidazolium/HCl buffer, 1 mM EDTA, pH 7.5. The figures are the result of at least two independent determinations. The values determined in this work for mandelate are identical to those given in (*28*).

[a] From Ref. (*28*).

essentially due to the high K_M value of the latter. HYPAH is still a somewhat better substrate than L-lactate.

In order to see if recognition of the hydroxamate function is specific to HAO, we tested the inhibitory capacity of HYPAH with respect to the flavodehydrogenase domain of flavocytochrome b_2 (FDH), using lactate as the substrate. Indeed, L-mandelate is known to be an inhibitor as well as a very poor substrate for Flb2 and its FDH (*37,38*). Its specific activity with respect to Flb2 is 4 orders of magnitude lower than that of L-lactate. In the present case, both compounds gave similar series of intersecting lines in the Dixon plot; however, when the data were replotted according to Cornish-Bowden (*33*), the mandelate data gave a series of parallel lines (not shown), confirming that is is a purely competitive inhibitor, whereas the lines for HYPAH were intersecting in the upper left quadrant, indicating this compound exerts a mixed inhibition. The plots for HYPAH are given in Fig. 2. This mixed inhibition is reminiscent of that of pyruvate and oxalate with respect to Flb2, a behaviour which has been ascribed to the fact that pyruvate binds at the active site when the flavin is in the oxidized state as well as when it is in the semiquinone state (*39–43*). The respective K_i values are presented in Table 2. HYPAH affinity in the EI complex (K_{ic}) is lower than that of mandelate by about two orders of magnitude; this difference is similar to that found between the respective K_M values for HAO. We did not test HYPAH as a potential substrate, because it appeared to react with ferricyanide in the absence of enzyme.

pH Profiles of the HAO Kinetic Parameters for Mandelate and Its Hydroxamate

The Flb2 crystal structure showed that the reaction product, pyruvate, has its carboxylate within hydrogen bonding distance of Tyr143 and Arg376 (*7*) (Fig. 1). Subsequent modeling studies, combined with site-directed mutagenesis studies, led to the idea that the carboxylate group of the substrates bind to these same side chains, as well as probably to that of Arg289 (*12,15,44–47*). In HAO, these residues are Phe23, Arg164, and Arg250. Mandelic acid has a pK_a of 3.8 (*48*) and we can assume it binds in the ionized state, which should be well stabilized by the electrostatic interaction with the two arginines. We titrated HYPAH and found a pK_a of 9.1, well within the range found for hydroxamates (*49*). One may thus ask what is the ionization state of the bound hydroxamic acid. In order to answer the question, we undertook a study of the influence of pH on the activity of HAO, both for mandelate, since no data were available, and for its hydroxamate.

These studies were carried out with a different buffer system (see Materials and Methods). With HYPAH, no measurements could be carried out at low pH, because it appeared to react with DCIP in the absence of enzyme at pH 6 and below. The results are shown in Figs. 3 and 4 for k_{cat} and k_{cat}/K_M, respectively, and the deduced pK_a values are given in Table 3. In all cases, bell-shaped profiles are observed; as the pH increases, the deprotonation of a first group increases the activity, and the deprotonation of a second group decreases it. With mandelate, a broad optimum is observed for k_{cat} between pH 7 and pH 9 (Fig. 3). The pH optimum is narrower for HYPAH, lying between pH 8 and 8.5, so that at the respective pH optima, the two compounds are turned over at the same rate. The optimal k_{cat}/K_M values differ by less than an order of magnitude, and this stands in contrast with the 100-fold difference

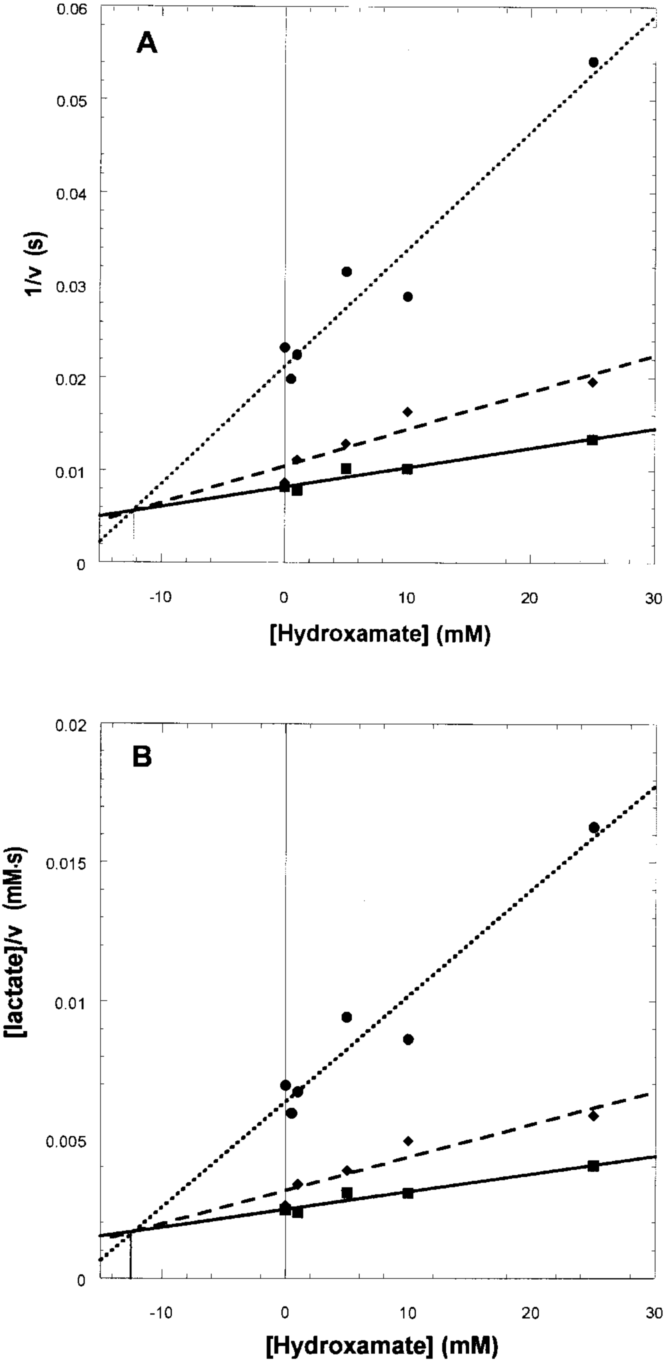


FIG. 2. Inhibition of the FDH with HYPAH. L-Lactate was used as the substrate at 0.1 mM (dotted line), 0.3 mM (dashed line), and 0.6 mM (solid line). Assays were carried out at 30°C in 0.1 M phosphate buffer, pH 7.0, with 13 mM ferricyanide as acceptor. (A) fitting to Eq. [2]; (B) fitting to Eq. [3].

TABLE 2

Inhibition of the FDH

Inhibitor	K_{ic} (mM)	K_{iu} (mM)
L-Mandelic acid	0.09 ± 0.02	—
HYPAH	12.2 ± 0.1	12.2 ± 0.1

Note. The experiments were performed at 30°C in 0.1 M phosphate buffer, 1 mM EDTA, pH 7, using 13 mM ferricyanide as acceptor. The parameters were obtained by fitting the data to Eqs. 2 and 3.

found at pH 7.5, in imidazolium buffer (Table 1). This difference arises essentially from effects on K_M values; they are lower for mandelate and higher for HYPAH in imidazolium buffer compared to the buffer used for the pH-rate studies. The reason for this difference is unclear. In the HYPAH k_{cat} versus pH profile, the acidic pK_a appears to be displaced upwards by about one pH unit, whereas the basic pK_a is the same as that for mandelate. The k_{cat}/K_M versus pH profile for mandelate shows the same flat optimum as the k_{cat} profile, with identical pK_a values (Fig. 4). For the hydroxamate, the curve is again sharper, and with a higher acidic pK_a (identical within error to that for the k_{cat} profile) and a lower basic pK_a.

Inhibition studies with HAO. The results are presented in Table 4. Phenylacetate,

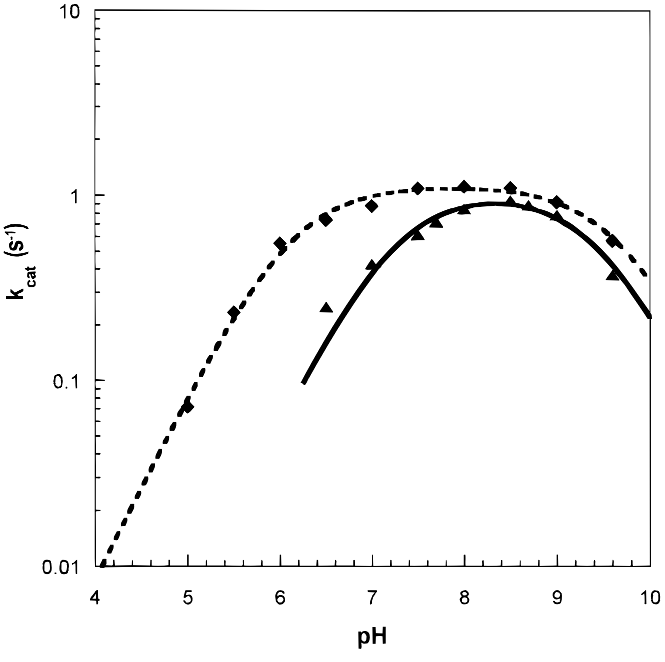


FIG. 3. pH dependence of k_{cat}. L-Mandelate (dashed line); HYPAH (solid line). The values were determined at 30°C in the universal buffer (see Materials and Methods).

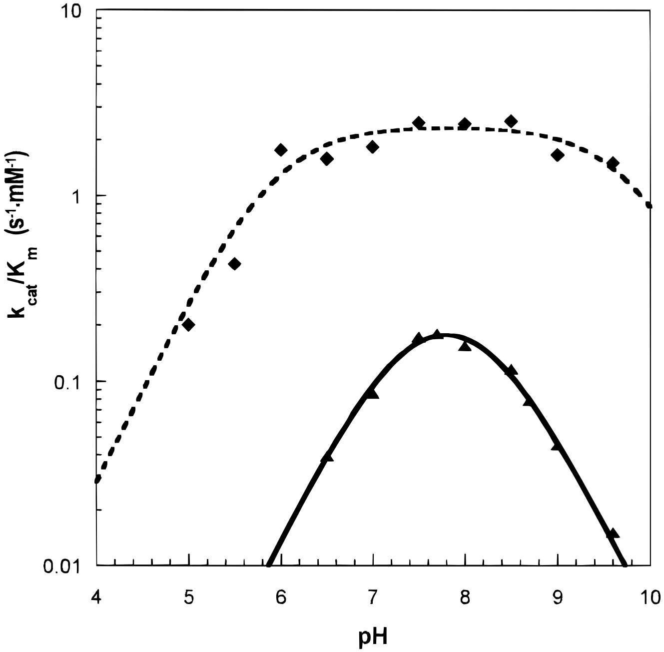


FIG. 4. pH dependence of k_{cat}/K_M. L-Mandelate (dashed line); HYPAH (solid line). The values were determined at 30°C in the universal buffer (see Materials and Methods).

benzoylformate –the mandelate oxidation product– and benzohydroxamate all behaved as competitive inhibitors. The best of the three is benzoylformic acid, with an affinity about 3-fold lower than that of the substrate. The worst is phenylacetic acid. Its K_i is about 200-fold lower than the mandelate K_M value. This suggests that the substrate 2-hydroxyl group contributes by about 3.2 kcal/mol to the binding energy, probably through a hydrogen bond to Tyr129, which orients the C2 substituents in the active site, as was suggested in the study of the Tyr254Phe mutant Flb2 (*30,37*). Similarly,

TABLE 3

pH Dependence Parameters

	k_{cat}		k_{cat}/K_M			
Substrate	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	k_{opt} (s^{-1})	$(k_{cat}/K_M)_{opt}$ ($mM^{-1}s^{-1}$)
L-Mandelic acid	6.1 ± 0.1	9.6 ± 0.3	5.9 ± 0.2	9.8 ± 0.3	1.1 ± 0.1	2.4 ± 0.2
HYPAH	7.2 ± 0.1	9.4 ± 0.1	7.3 ± 0.1	8.3 ± 0.1	1.0 ± 0.1	0.3 ± 0.1

Note. The values were determined at 30°C in universal buffer (see Materials and Methods). Assays were performed by monitoring the absorbance of DCIP at 600 nm. The values presented are deduced from nonlinear regression analyses according to Eq. [4]. These analyses yield pK_a values as well as k_{opt} and $(k_{cat}/K_m)_{opt}$, which are the respective values at the curve maximum.

TABLE 4

Inhibitors of HAO

Inhibitor	K_i (mM)
Benzoylformic acid	0.52 ± 0.2
Benzohydroxamic acid	12.5 ± 0.3
Phenylacetic acid	21.4 ± 0.3

Note. Experimental data were obtained at 30°C, 0.1 M imidazolium/HCl buffer, 1 mM EDTA, pH 7.5. The values were calculated by fitting the data to Eq. [2]; the competitive nature of the inhibition was verified using Eq. [3], which yields parallel lines for competitive inhibition.

the binding affinities of L-lactate and propionate for WT Flb2 differ by about two orders of magnitude (*50*).

DISCUSSION

2-Hydroxy Hydroxamic Acids as Substrates

Cromartie and Walsh (*36*) determined an isotope effect of 8 with L-2-[^{2}H]lactate in the steady state. Lactate is a slow substrate for HAO (Table 1); with the faster mandelate, we have preliminary evidence that the rate of flavin reduction in a stopped-flow apparatus is only about two-fold higher than the steady-state rate with the same substrate. These results indicate that flavin reduction is the main rate-limiting step in the mandelate catalytic cycle, and we thus feel justified in assuming that K_M values are a good reflection of dissociation constants.

All substrates, physiological or not, known for members of the family of FMN-dependent 2-hydroxy acid-oxidizing enzymes carry a carboxylate group. This paper describes for the first time a substrate with a different function. Binding is not related to the substitution of Tyr with Phe at HAO position 23, since HYPAH is also recognized by Flb2 and its K_i value (Table 2) differs from that of mandelate by a factor very similar to the K_M values difference between these compounds as HAO substrates (Table 1). It is thus likely that hydroxamic acids can be ligands for other family members, and substrates as well. The respective k_{cat} values for mandelate and its hydroxamic acid are practically identical (Tables 1 and 3), indicating that the $\Delta\Delta G$ values between ES and ES‡ complexes is identical for both substrates.

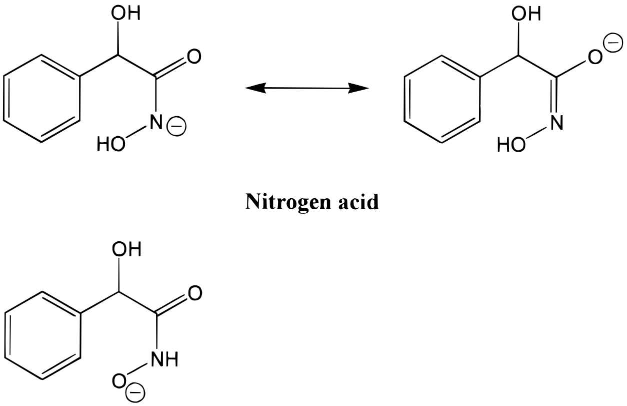
Hydroxamic acids such as HYPAH and benzohydroxamic acid are considered to exist in the keto form in solution. The C, O and N atoms of the C(O)-NHOH group are expected to lie in one plane, by analogy with amide and carboxylate groups, but the oxygen on nitrogen could adopt two different positions relative to the carbonyl group (*51*) (Scheme 2). The orientation shown for **2** might induce a binding mode rather different from that of the carboxylate and possibly displace other substrate atoms, in particular C2, whereas, with the orientation as in **1**, the nitrogen might occupy the same location as the bottom oxygen in Fig. 1, and its bound oxygen could possibly point out into the solvent, since Phe23 (Tyr143Flb2) lies at the opening of the active site.

There has been discussion as to which is the acidic hydrogen in a hydroxamic

Neutral form, positional isomer.

1 **2**

Ionized form.



Nitrogen acid

Oxygen acid

SCHEME 2.

acid (*51*). These compounds are now generally considered as oxygen acids, but benzohydroxamic acid is predominantly a nitrogen acid in methanol (*49,52*). If, as we think (see next section), HYPAH binds in its ionized form, the mesomeric form of the nitrogen acid shown in Scheme 2 would present substantial similarity to a carboxylate. This raises the question of the lower affinity of HYPAH compared to mandelate, for the two enzymes tested. Replacing the carboxylate with the hydroxamate function appears to have about the same effect on the affinity for the free enzyme and for the ES‡ complex, as judged from the optimal K_M and k_{cat}/K_M values, respectively (a factor of about 10^2 in imidazolium buffer, of about 10 in the universal buffer) (Tables 1 and 3 and Fig. 4). The additional oxygen in the hydroxamic acid function relative to a carboxylate might alter the binding mode more than predicted above and therefore perturb the electrostatic interactions with the active-site arginines, leading to a less efficient stabilization of the negative charge, which is important both for binding and for catalysis of a reaction involving a carbanion. It may be for example that the additional hydroxyl group prevents a movement of Arg164 toward the ligand; such a movement has been observed in the Flb2 crystal (*45,53*) and its importance studied by site-directed mutagenesis (*44,46*). Modeling studies, when the HAO refined coordinates become available (*9*), might shed some light onto the matter.

Does the Hydroxamic Acid Bind in the Neutral or the Ionized Form?

In order to answer the question, the influence of pH on the catalytic behaviour of HAO with respect to a classical substrate such as mandelate has to be examined first. Considering that mandelate is expected not to change its ionization state over the pH range examined, the results of our study indicate that two enzyme ionizations are catalytically influential: that of an acidic group with a pK_a around 6 and that of a basic group with a pK_a around 9.6–9.8 (Table 3 and Figs. 3 and 4). For reasons given in the preceding section, we feel justified in considering that the pH-rate profiles determined for steady-state parameters are a good reflection of those for flavin reduction, the main or only rate-limiting step, depending on the substrate. Table 5 recalls values determined for two homologous enzymes, namely Flb2 (*54,55*) and MDH (*5,56*); in both cases, similar bell-shaped curves were obtained. With Flb2, pK_a values were identical for flavin reduction, heme reduction and in the steady-state (*55*), with flavin reduction being the main rate-limiting step (*57*). For this enzyme, the acidic pK_a value seemed to be somewhat lower and the basic one somewhat higher in the ES complex (k_{cat} profile) than in the free enzyme (k_{cat}/K_M profile). For MDH, the basic pK_a value also increased upon complexation, whereas the acidic pK_a value did not appear to be significantly influenced by substrate binding. For HAO, the values are identical in both mandelate profiles, and on the whole similar to those presented for the two other enzymes.

In view of active-site similarities (Fig. 1), it seems legitimate to conclude that the kinetic pK_a values determined with normal substrates for the three enzymes reflect the ionization of the same protein side-chains. The acidic pK_a value is the easiest to ascribe. In early studies, it was reasonably proposed to be that of a histidine (*55*); the crystal structures (*8–10*) and site-directed mutagenesis studies (*58–60*) indicate it to be His373(Flb2) and its homologues in HAO, GOX, MDH, and LOX (Fig. 1). A pK_a value between 5 and 6 is reasonable for an active site base. Why it should be higher in HAO than in the two other enzymes is probably due to local differences.

TABLE 5

p*K*a values for Flb2 and MDH

	k_{cat}		k_{cat}/K_M	
Enzyme	pKa_1	pKa_2	pKa_1	pKa_2
Flb_2[a]	5.30	9.7	6.0	8.8
MDH, WT[b]	5.1 ± 0.1	9.6 ± 0.1	5.5 ± 0.1	8.9 ± 0.1
MDH, WT[c]	4.8 ± 0.1	10.3 ± 0.1	5.1 ± 0.1	9.7 ± 0.1
MDH, R277K[c]	5.1 ± 0.1	9.9 ± 0.1	4.9 ± 0.1	9.4 ± 0.1

[a] Taken from (*55*). The values were found to be identical in rapid-kinetics studies for flavin and heme reduction and in the steady state for ferricyanide reduction.

[b] Taken from (*5*); the values were determined in the same buffer we used (see Materials and Methods).

[c] Taken from (*56*); the buffer system was different.

The only obvious one is the Tyr to Phe replacement at position 23 in HAO; but it is difficult to predict what influence this could have, if any.

The identity of the basic group is more difficult to predict. Groups potentially ionizing in the basic pH range in the catalytic site and its vicinity for Flb2 are Tyr143 and 254, Arg289, Lys349, and Arg376 (the corresponding numbering in HAO and MDH is given in the legend of Fig. 1). In addition, one might consider the ionization of FMN N3. In MDH it appears to occur at high pH (*5*), whereas in the HAO isozyme β1 used in this study the pK_a at N3 is 7.9 (*28*). This is much lower than the value of 9.6 predicted for free HAO from the mandelate k_{cat}/K_M profile. Furthermore, we have preliminary evidence that binding of a negatively charged ligand raises the N3-H pK_a (D. Amar, unpublished results), and one would thus expect a basic pK_a difference between the k_{cat} and k_{cat}/K_M profiles. Therefore, flavin ionization at N3 does not appear to influence the enzyme kinetics. For HAO, position 23 is a phenylalanine and not a tyrosine as in the other enzymes (Fig. 1), and thus, for the other family enzymes, an influence of the ionization of the homologous tyrosine (Fig. 1) can be excluded. On the basis of inhibition studies with substrate analogues, Lehoux and Mitra (*5*) suggested that the basic group, the deprotonation of which lowers the enzyme activity, is one of the active site arginines. While this idea appears to make good sense at first sight in view of the results presented, some objections come to mind. First, a free arginine side chain is credited with a pK_a of 12.5; although some spectacular examples of pK_a changes in proteins have been described (*61*), which are due to the protein environment, there is no undoubted case reported for arginines, to our knowledge. Second, if one examines the crystal structures of Flb2 (1FCB) and GOX (1GOX), in both free active sites Arg289/164 is interacting with invariant Asp292/167, and such an electrostatic interaction should increase rather than decrease the pK_a of this arginine in the free enzyme. What about Arg376/277 (Fig. 1)? If the basic pK_a for Flb2 and MDH belongs to this residue, one would expect its pK_a to be raised upon interaction with the substrate carboxylate; this is indeed experimentally observed for the wild-type enzymes (Table 5); but Arg277 was mutated to a lysine in MDH, and within error the pK_a values were identical to those of the wild-type enzyme (*56*), which was unexpected. Thus, the authors concluded that the identity of the MDH group with a pK_a of 9.7 had still to be unequivocally determined. This conclusion must be extended to other family members.

At this point of the reasoning, if the kinetically determined basic pK_a can be ascribed to a specific group which would not be Arg376(Flb2) and its homologues, the remaining candidates are Tyr254(Flb2) and Lys349(Flb2) (Fig. 1). According to a short communication on Flb2 tyrosine mutants (*62*), Tyr254 can probably be eliminated as well. Lys349 (and its homologues in GOX and HAO) lies in a hydrophobic environment, and in the oxidized enzymes interacts with neutral groups (FMN N1 and O2, ribityl chain O2′) (*10*). Thus, its pK_a could be lowered from 10.5 for free lysine to around 9 in the free enzymes. Its becoming neutral would be detrimental to the binding of negatively charged substrates; reciprocally, their binding would be expected to raise its pK_a, as observed for Flb2 and MDH (Table 5); the lysine ionization would also be detrimental to catalysis, since its positively charged side chain is one of the elements contributing to the oxidized FMN electron deficiency and it stabilizes the anionic N1 of reduced flavin, thus facilitating flavin reduction. In this respect, it is interesting to note that the mutation in LMO of Lys266 (349Flb2) to Met yielded, to the authors' surprise, an enzyme with strongly impaired affinity for L- and D-lactate, as well as for oxalate (*63*), among other consequences on catalysis, The effect of pH on enzyme functioning was not studied by the authors.

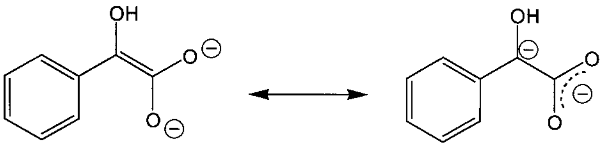
If we now turn our attention to the pH profiles for HYPAH, it seems reasonable to assume that their differences with the corresponding profiles for mandelate can be ascribed to the hydroxamic acid ionization which, with a pK_a of 9.1 for the free compound, occurs in the pH range studied. The fact that it was impossible to obtain rates below pH 6.5 does not facilitate the analysis. The k_{cat} profile appears to be the easiest to interpret. Within error, the basic pK_a, ascribed to the ES complex, appears to have the same value as that determined for mandelate (Fig. 3 and Table 3). We want to suggest that the increase in the apparent acidic pK_a is connected to the substrate ionization. With the limited data available, we cannot decide if the slope of the acidic descending limb is one, as it might seem, in which case we might be missing another lower pK_a or if it is two, in which case the value might be intermediate between those for the histidine and the hydroxamic acid group ionizations. Both these interpretations assume that HYPAH binds in its ionized form, and that its pK_a in the Michaelis complex is probably lowered relative to that in the free state. This hypothesis is reasonable in view of the presence of two arginine residues in the active site which may interact with the hydroxamate as they do with carboxylates, and that the interaction would involve charged groups on all partners (*64*). If the catalytically competent form of the hydroxamic acid were the neutral one, since it has a pK_a value very similar to that of the basic enzyme group, it is not clear why its ionization should raise the acidic pK_a. In contrast, the binding of a neutral substrate should destabilize the protonated histidine, and one would expect its pK_a to be lowered rather than raised.

The k_{cat}/K_M profile for HYPAH cannot be interpreted in a straightforward manner, considering that the separation between the experimental values is only on the order of one pH unit (*64–66*). This profile is expected to reflect both free enzyme and free substrate ionizations. If we consider the free enzyme pK_a values given by the mandelate profile (Fig. 4), and the free HYPAH pK_a of 9.1, the inward shift of both acidic and basic limbs is totally unexpected. One would expect no change of the acidic limb, perhaps a shift of the basic pK_a towards a value intermediate between 9.1 and 9.8, with a slope change. Usually quoted complicating factors for pH-rate profiles are sticky substrates, or a change in rate-limiting step (*64–66*). We cannot rigorously rule out either of these factors; nevertheless, in our case, neither sounds like a plausible hypothesis. That the hydroxamic acid should be a sticky substrate, with $k_{off} << k_2(k_{cat})$ seems unlikely, considering that k_{cat} itself is quite small (Table 1). In addition, a change in rate-limiting step would mean that flavin reoxidation by DCIP would have become rate limiting; this hypothesis also seems unlikely, unless the product were sticky and prevented electron transfer to the acceptor. Thus, at this stage, the k_{cat}/K_M profile for HYPAH remains puzzling. It is on the basis of the interpretation of the k_{cat} profile that we favor the interpretation that HYPAH binds in the ionized form and that its pK_a is lowered in the ES complex; but clearly, more evidence is required for an unambiguous conclusion.

Benzohydroxamate Does Not Behave as a Transition State Analogue

The hydroxamate function has been used in a number of inhibitors of enzymes that abstract a proton from a carbon adjacent to a carbonyl or carboxylate group. The first example described was that of phosphoglycolohydroxamate with triose phosphate isomerase, which turned out to display a K_i value lower than the dihydroxyacetone phosphate K_M value by two orders of magnitude (*25*). It was proposed that this remarkable affinity was due to the similarity between a mesomeric form of the ionized hydroxamic acid and the substrate enediolate intermediate on the catalytic pathway. Subsequent crystallographic studies confirmed that the hydroxamate was bound as expected (*67*). For a number of other enzymes, replacing a carbonyl or a carboxylate with a hydroxamate function also had important effects on affinity (*26,68*). In particular, benzohydroxamate was found to inhibit mandelate racemase with an affinity about 100-fold higher than that of mandelate (*27*). The structure and mechanism of this enzyme have been studied in detail (*69*), and it is clear that an "enolic intermediate" lies on the reaction pathway, although its exact nature, enol or enolate, has been debated (*70–72*). Gerlt and Gassman (*70,71*) have underlined that a hydrogen on a carbon adjacent to a carboxylate has a very low acidity and that enzymes could facilitate the reaction by using a concerted mechanism which would stabilize an intermediate enolic form of the carbanion (shown unprotonated in Scheme 3). Benzohydroxamic acid was proposed to bind as a mimic of the dianionic *aci*-carboxylate tautomer of mandelate carbanion, namely as the O-deprotonated form of the benzohydroxamic acid enol, which would present similarities to the transition state structure (*27*) (Scheme 3).

As both mandelate racemase and HAO transform mandelate and abstract the substrate C2 proton in the first catalytic step, we tested benzohydroxamate as an inhibitor.



Mandelate carbanion

Benzohydroxamate N-acid enolate **Benzohydroxamate O-acid**

SCHEME 3.

Its K_i value at pH 7.5 is nearly two orders of magnitude higher than the substrate K_M value; the pK_a of the free compound is 8.7 (*73*), not very different from that of HYPAH. Pending further binding studies as a function of pH, it seems that benzohydroxamate has much less affinity for HAO than the substrate and its product, benzoylformic acid (Table 4), and an affinity similar to that of HYPAH. If it behaved as an analog of a putative *aci*-carboxylate intermediate, it could have the structure shown in Scheme 3 (N-acid enolate), proposed for mandelate racemase (*27*). In view of our results, this structure does not appear to be specifically stabilized; alternatively, HYPAH could bind as the benzohydroxamate O-acid (Scheme 3), which appears to be the preferred ionized form in aqueous solution (*49*). We have reasons to believe that the affinities of mandelate and HYPAH are in large part dictated by the aromatic ring, which restricts possible locations for C2. As shown in Schemes 2 and 3, HYPAH would have one more atom than mandelate at the carboxylate position, and benzohydroxamate one less. Obviously, benzohydroxamic acid does not fit better in the active site than HYPAH.

Altogether, our results do not provide evidence that the transient carbanion on the HAO catalytic pathway is stabilized by electron delocalization towards the carboxylate. We are fully aware that they do not, however, rule out this possibility, even if they do not support it. Indeed, xylarohydroxamate, an analog of a 5-carbon substrate for D-glucarate dehydratase, was found to bind with an affinity only similar to that of the substrate, and yet it is clear that the reaction mechanism passes through an enolic intermediate (*74*).

It is clear that the factors important for binding are very different in mandelate racemase and HAO; in the first case the carboxylate interacts with a glutamic acid and a Mg^{2+} ion, which also interacts with the hydroxyl group (*69*). In the second case, the carboxylate is stabilized by interactions with two arginine residues, and the hydroxyl group is hydrogen bonded to a tyrosine (*7,37,44,45,53*) (Fig. 1). Nevertheless, the very different behaviour of benzohydroxamate with the two enzymes may also arise from different catalytic requirements. We want to stress that, in the case of the FMN-dependent hydroxy acid-oxidizing enzymes, an *aci*-carboxylate-like transition state is not an attractive idea. Why should the enzyme contribute to delocalize the electrons the substrate has to lose? A strong electrostatic stabilization of the carboxylate, as provided by these enzymes, would contribute to decrease the pK_a of the bound substrate C2-proton. Furthermore, we have shown that the active-site histidine in Flb2 has a higher pK_a in the reduced state than in the oxidized state (*75,76*). The increase might start being felt in the transition state if it were a late one and would thus contribute to facilitate proton abstraction. Finally, the proximity of electron deficient flavin makes carbanion stabilization without object. And indeed, since C2-hydrogen abstraction is rate limiting for flavin reduction in these enzymes, the subsequent electron transfer must be very rapid, since no intermediate has ever been detected in rapid-kinetics studies for any of the homologous enzymes.

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