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STUDIES ON THE MECHANISM OF INHIBITION OF REDOX ENZYMES BY SUBSTITUTED HYDROXAMIC ACIDS

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Summary

Substituted primary hydroxamic acids were found to inhibit the catalytic activity of a number of redox enzymes. The inhibition was not related to the nature of the metal-active site of the enzyme nor to the nature of the oxygen-containing substrate. Two easily available enzymes, mushroom tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) and horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7), which were potently inhibited by hydroxamic acids, were chosen for more detailed study.

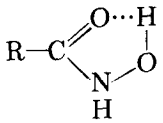
A kinetic analysis of the inhibitory effects on the partially purified tyrosinase of mushroom (*Agaricus bispora*) revealed that inhibition was reversible and competitive with respect to reducing substrate concentration, but was not competitive with respect to molecular oxygen concentration.

A spectrophotometric and EPR study of the binding of salicylhydroxamic acid to horseradish peroxidase revealed that this hydroxamic acid was bound to the enzyme in the same manner as a typical substrate, hydroquinone. Spectroscopic and thermodynamic measurements of the binding reactions suggested that this binding site is close to, but not directly onto, the heme group of the enzyme.

From these results it is concluded that the mode of inhibition of hydroxamic acids need not be, as generally supposed, by metal chelation, and mechanisms involving either hydrogen bonding at the reducing substrate binding site or the formation of a charge transfer complex between hydroxamic acid and an electron-accepting group in the enzyme are considered to be more feasible. The relevance of these findings to deductions on the nature of other hydroxamic acid-inhibitable systems is discussed.

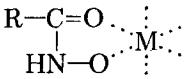
Introduction

The primary hydroxamic acids, of the general structure R-(CO)NHOH, have been well studied by physical means. For example, infrared spectroscopy [1] has revealed that the monomeric molecules are intramolecularly hydrogen bonded, thus:



This structure may be replaced by intermolecular hydrogen bonds under appropriate conditions [1].

A further property of the hydroxamic acids is their ability to chelate metal ions, presumably as typical bidentate oxygen donors [2—4], thus:



Chelation of iron in the ferric state is particularly favored [4]. This iron-chelating property has been exploited in a chemotherapeutic treatment for removal of excess iron from Cooley's anemia patients [5,6].

In 1971, Schonbaum et al. [7] reported the specific inhibition by primary hydroxamic acids of the cyanide- and antimycin A-insensitive alternative respiratory pathway which is present in many higher plant mitochondria (for example, see review in ref. 8). Later, however, it was shown that hydroxamic acids could also form complexes with horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) and that the complexing was competitive with respect to reducing substrate [9]. Recently, an inhibitory effect on the L-α-glycerophosphate oxidase activity of *Trypanosoma* sp. has been demonstrated [10]. These inhibitory properties of the hydroxamic acids have sometimes been attributed to their metal-chelating ability and in the case of the higher plant alternative oxidase, many workers have even taken the hydroxamic acid effect as evidence of an iron-sulfur center involvement in the pathway.

In this report, we show that hydroxamic acids are also capable of inhibiting tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) reactions, and at concentrations much lower than those required for inhibition of the alternative pathway. Kinetic, spectroscopic and thermodynamic evidence is presented that the mechanism of inhibition of both tyrosinase and horseradish peroxidase is one of competition with the reducing substrate for its binding site. This binding may be close enough to the metal of the active site so that it perturbs its environment, but is probably not directly onto the metal atom, i.e. the hydroxamic acids may not act by metal chelation in these systems.

These notions may be generalized to include other hydroxamic acid-inhibitable systems which we are not capable of examining so directly. In particular, their relevance to studies of the mitochondrial alternative oxidase of higher plants [8] is discussed.

Materials and Methods

Enzyme assays. Tyrosinase (from mushroom, grade II from Sigma, monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) was assayed in a medium containing sodium citrate at a pH of 5.6 and 25°C [11]. Substrate concentrations were varied up to a maximum of 0.1 M (pyrogallol), 3.3 mM (L-tyrosine) or 2 mM (epinephine). Oxygen consumption rates were measured with a Clark-type oxygen electrode and maximal rates were used.

Peroxidase (from horseradish, Sigma grade VI, donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) and cytochrome *c* peroxidase (from yeast, EC 1.11.1.5, the kind gift of Dr. T. Yonetani), were assayed in a medium containing 40 mM potassium phosphate and 1 mM EDTA at a pH of 7.0. The oxidation of 0.033% *p*-phenylenediamine at 485 nm or of 40 μM reduced cytochrome *c* at 550 nm was monitored after initiation of the reaction with 0.001% hydrogen peroxide.

Catalase (Boehringer, from beef heart, EC 1.11.1.6) was assayed in a medium containing 40 mM potassium phosphate and 1 mM EDTA at a pH of 7.0 and which had been deoxygenated with argon gas. The initial rate of oxygen generation on addition of 0.125 mM hydrogen peroxide was monitored with a Clark-type oxygen electrode.

Cytochrome *c* oxidase (EC 1.9.3.1) activity of isolated mitochondria was assayed in a standard mitochondrial preparation [12] by measurement of the rate of oxygen consumption on addition of 10 mM ascorbate/0.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The suspension medium was 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate at pH 7.2.

Ascorbic acid oxidase (from zuccini squash, EC 1.10.3.3) was a gift of Dr. C.R. Dawson and was assayed in a medium containing 40 mM potassium phosphate and 1 mM EDTA at a pH 7.0. Oxygen consumption rate was monitored with a Clark-type oxygen electrode after addition of 1 mM neutralized ascorbic acid.

Xanthine oxidase (from buttermilk, EC 1.2.3.2, purchased from Sigma Chemical Co., grade I) was assayed from its rate of superoxide anion generation in the presence of 0.1 mM xanthine and generation rate was monitored from the rate of production of adrenochrome from 1 mM epinephrine by measuring the absorbancy change at 485 minus 575 nm ($\epsilon = 3.97\ mM^{-1} \cdot cm^{-1}$).

Glycollate oxidase (glycollate:O_2 oxidoreductase, EC 1.1.3.1) was assayed in spinach leaf mitochondria by the formation of glyoxylate phenylhydrazone by the method of Frierbend and Beevers [13], assayed aerobically by monitoring oxygen uptake. Approx. 100 μl sample (1—1.5 mg mitochondrial protein) was incubated in 2.5 ml reaction medium containing 50 mM triethanolamine buffer, pH 7.9, 4 mM EDTA, 0.001% Triton X-100, 60 μM KCN and 24 μM FMN. Following incubation in the dark for 5 min, the reaction was initiated by addition of 12 mM glycollate and the rate of oxygen consumption was determined.

Superoxide dismutase (EC 1.15.1.1, from bovine blood, purchased from Sigma Chemical Co., Type I), was assayed either by the xanthine/xanthine oxidase [14] or the $K^+O_2^-$ [15] assay. In the xanthine assay, superoxide dismutase activity was determined at 560 nm by following the reduction of nitroblue tetrazolium to formazan. In the $K^+O_2^-$ assay, superoxide was provided by addi-

tion of 200 μl dimethylsulfoxide saturated with potassium superoxide to 2.8 ml medium containing 56 μM nitroblue tetrazolium, 10 μM EDTA and 10 mM phosphate buffer at pH 7.5. Absorbance was read at 560 nm as above. Varying amounts of enzyme (10—200 μl) were used in order to obtain a standard curve of percentage inhibition versus enzyme concentrations.

The possible effect of salicylhydroxamic acid upon rat liver microsomal cytochrome *P*-450 was determined by monitoring the effect of the reagent on the visible and EPR spectrum of the native oxidized enzyme. Other effectors of the cytochrome *P*-450 have already been shown to produce spectra shifts of the hemoprotein [16,17]. A lack of effect was taken as indicative of a lack of interaction of the hydroxamic acid with the hemoprotein.

EPR measurements. Samples for EPR measurements were frozen in liquid nitrogen in quartz EPR tubes and stored at 77 K until assayed. Spectra were obtained with a Varian E-4 spectrometer (Varian Associates). The temperature of the samples for EPR measurements was controlled with a variable temperature cryostat (Air Products Model LTD-3-110). Temperature was monitored with a calibrated carbon resistor placed in the helium flow directly below the sample and *g*-values were corrected by reference to a weak pitch standard.

Redox potentiometry. The method used was that of Dutton [18]. The sample was 0.25 mg/ml horseradish peroxidase in an appropriate buffer, kept anaerobic with a constant flow of argon gas. Mediators used were benzyl viologen (10 μM), indigo 2-sulfonate (10 μM), 2-hydroxy-1,4-naphthoquinone (10 μM), anthraquinone-2,6-sulfonate (10 μM) and phenosafranine (5 μM). Reductive and oxidative titrations were by additions of solutions of sodium dithionite and potassium ferricyanide, respectively. Potential was measured with a platinum electrode with a standard saturated KCl calomel half cell. The redox state of the enzyme was monitored at 435—450 nm. The absorbance changes caused by the dyes were titrated in separate experiments and were subtracted from the peroxidase titrations.

Chemicals. m-Chlorobenzhydroxamic acid was the kind gift of Dr. G.R. Schonbaum and a sample of purified ascorbic acid oxidase was generously supplied by Dr. C.R. Dawson.

Protein. Protein was assayed by the method of Lowry et al. [19] with bovine serum albumin as standard.

Results

A number of redox enzymes were tested to determine whether there was a possible common factor which could account for the observed effects of the hydroxamic acids. These results, which were obtained by experimentation or by reference to the available literature, are presented in Table I. Salicylhydroxamic acid at 1 mM concentration was used for the tests and the results obtained were that of either very potent inhibition (>90% inhibition) or of no inhibition at all (sometimes even a slight stimulation).

The points to note from this survey are that there is no apparent relation between the potential for hydroxamic acid inhibition and either the nature of the oxygen-binding metal atom (e.g. copper or iron) or the nature of the oxygen substrate (i.e. either molecular oxygen, hydrogen peroxide or super-

TABLE I

THE INHIBITORY PROPERTIES OF SALICYLHYDROXAMIC ACID ON A VARIETY OF REDOX ENZYMES

Enzymes were assayed as described in Materials and Methods. The enzymes were preincubated with 1 mM salicylhydroxamic acid before the (reducing) substrate was added. In cases where non-linear rates were observed, the initial reaction rate was always taken.

Enzyme	Source	Inhibition by 1 mM salicyl-hydroxamic acid	Presumed enzyme-active site	Source of information on inhibition
Tyrosinase	Mushroom	+	Copper	By experiment
	Potato tuber	+	Copper	By experiment
	Apple	+	Copper	By experiment
Ascorbic acid oxidase	Zuccini squash (*Cucurbita pepo*)	—	Copper	By experiment
Cytochrome oxidase	Rat liver	—	Copper, heme	By experiment
	Potato tuber	—	Copper, heme	Ref. 7
Higher plant alternative oxidase	Many sources	+	?	Ref. 7
Xanthine oxidase	Milk	— *	Molybdenum, FAD, FeS	By experiment
Glycollate oxidase	Spinach leaves	—	FAD	By experiment
Superoxide dismutase	Bovine blood	—	Copper	By experiment
L-α-Glycero-phosphate oxidase	*Trypanosoma* sp.	+	?	Ref. 10
Peroxidase	Horseradish	+	heme	By experiment
Cytochrome *c* peroxidase	Yeast	—	heme	By experiment
Catalase	Bovine liver	—	heme	By experiment
Lipoxygenase	Germinating seeds	+	?	Personal communication of Dr. C. Leopold
Cytochrome *P*-450	Rat liver	—	heme	By experiment

* Some inhibition at high concentration.

oxide anion). Furthermore, enzymes which are generally rather similar in terms of their metal active site (for example, catalase and horseradish peroxidase) are not necessarily affected equally by the inhibitors. These points are particularly emphasized in the case of cytochrome *c* peroxidase compared to horseradish peroxidase, where the former is unaffected and the latter potently inhibited by salicylhydroxamic acid. These enzymes both have heme-active sites and utilize hydrogen peroxide, and differ only in the nature of the substrate which donates reducing power (i.e. cytochrome *c* or a reduced quinone, respectively).

For further investigation, two enzymes which were potently inhibited by

hydroxamic acids were selected. These enzymes, mushroom tyrosinase and horseradish peroxidase, have already been characterized to a great extent and are easily available commercially in relatively large amounts.

The inhibitory effects of hydroxamic acids on partially purified mushroom tyrosinase

The experiments were performed with both a monohydroxy-(L-tyrosine) and a dihydroxy-(pyrogallol) substrate. It was found that maximum inhibition was not obtained immediately, particularly with partially inhibitory concentrations of inhibitors. Hence, before substrate was added, in each case the enzyme was incubated with inhibitor for 10 min at room temperature. This precaution made it easier to assess the maximum inhibition, even although the final inhibited rate was the same whether inhibitor was added before or after substrate. Fig. 1 illustrates the results obtained with three hydroxamic acids on the initial rate of oxidation of 3.3 mM pyrogallol by mushroom tyrosinase. All three inhibitors were effective in the less than micromolar range of concentration and the order of potency was *m*-chlorobenzhydroxamic acid (K_i apparent = 50 nM) > salicylhydroxamic acid (K_i apparent = 150 nM) > benzhydroxamic acid (K_i apparent = 600 nM) at a pyrogallol concentration of 3.3 mM. A similar order of potency of inhibition was obtained when L-tyrosine was the substrate and in this case the lag phase of onset of observable oxygen consumption increased as inhibition increased.

In order to investigate the inhibition further, the effects of salicylhydroxamic acid on mushroom tyrosinase oxidation of L-tyrosine was examined. When the experiments was performed at a variety of substrate and inhibitor concentrations and appropriate Lineweaver-Burk plots were drawn (Fig. 2), it was found that the inhibition was competitive with respect to L-tyrosine concentration. A further experiment was performed at fixed L-tyrosine and variable salicylhydroxamic acid concentrations and the reactions were allowed to

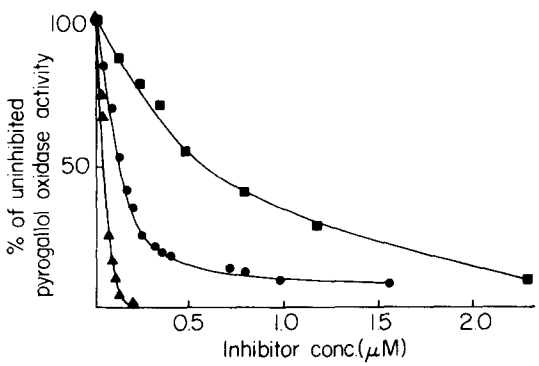


Fig. 1. The inhibition of mushroom tyrosinase by substituted hydroxamic acids. A suitable amount of mushroom tyrosinase (Sigma Chemical Co.) in 50 mM sodium citrate at pH 5.6 and 25°C was used. In each case the reaction was initiated by the addition of pyrogallol and the initial rate of oxygen consumption was taken as a measure of rate of reaction. When assays were performed with inhibitor, the enzyme was preincubated with inhibitor for 10 min, so that maximum inhibition was always obtained. Symbols are: ▲———▲, *m*-chlorobenzhydroxamic acid; ●———●, salicylhydroxamic acid; ■———■, benzhydroxamic acid.

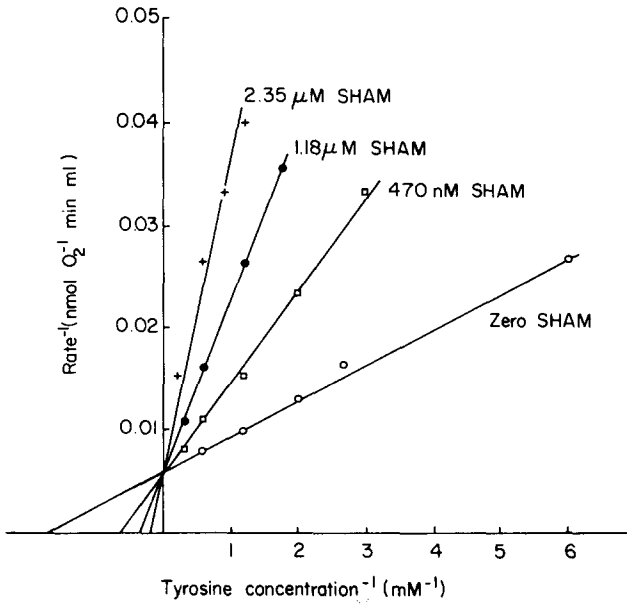


Fig. 2. Lineweaver-Burk plots of mushroom tyrosinase oxidation of L-tyrosine in the presence of inhibitor. The mushroom enzyme (125 μg/ml) in 50 mM sodium citrate at pH 5.6 and 25°C was incubated with inhibitor for 10 min before substrate was added. Sufficient time was allowed so that maximal oxygen consumption rate was observed. SHAM, salicylhydroxamic acid.

proceed to near zero oxygen tensions. Lineweaver-Burk plots of the inverse of reaction rates versus the inverse of oxygen concentrations revealed a rather more complex relation (uncompetitive) between oxygen and salicylhydroxamic acid concentrations viz. both the V of the reaction and its apparent K_m for oxygen decreased with increasing salicylhydroxamic acid concentrations (Fig. 3).

Similar experiments with pyrogallol as substrate did not yield conclusive results. This may be because the apparent K_m of the reaction for oxygen with pyrogallol as substrate was so high that linear reaction rates were never observed and hence a small change in apparent K_m for oxygen on addition of inhibitor would significantly alter the observed initial reaction rate. It has already been noted [20] that the apparent K_m of the tyrosinase reaction for oxygen varies with species and concentrations of reducing substrates.

The inhibition observed could not be imitated by 10 mM EDTA, nor was protection against inhibition given by 0.5 mM Cu^{2+} (in the form of $CuCl_2$), added prior to inhibition.

The reversible nature of the inhibitor was demonstrated by passing a solution of commercially purchased tyrosinase with 500 μM salicylhydroxamic acid (i.e. fully inhibited) through a Sephadex G-25 column (1.2 cm × 25 cm height), previously equilibrated with 50 mM sodium citrate buffer at a pH of 5.6. A fully active enzyme (in terms of specific activity per mg protein) was regained when the enzyme fraction was collected from the column effluent.

It was found, in agreement with other workers [21,22], that no significant amounts of EPR-detectable copper were present in the resting tyrosinase at any

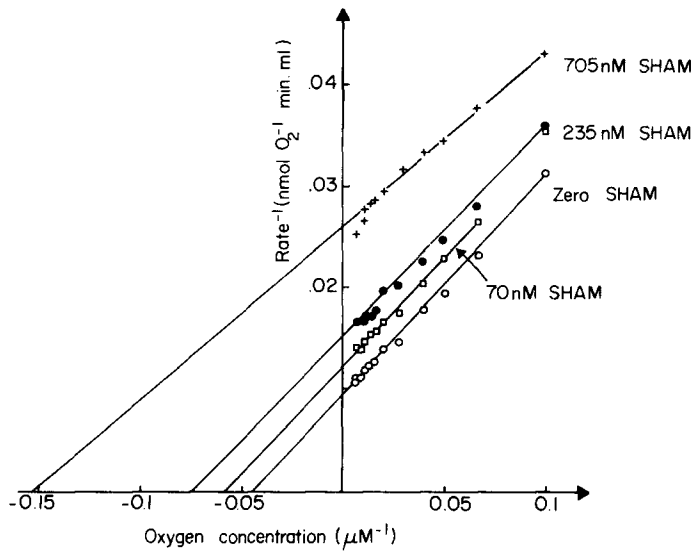


Fig. 3. Lineweaver-Burk plots of mushroom tyrosinase oxygen consumption at fixed L-tyrosine and variable inhibitor concentrations. The enzyme (125 μg/ml) was incubated in 50 mM sodium citrate at pH 5.6 and 25°C for 10 min with inhibitor before the substrate, 0.66 mM L-tyrosine, was added. The reaction was allowed to proceed to near completion and reaction rates were measured as a tangent to the oxygen consumption curve at appropriate oxygen concentrations. SHAM, salicylhydroxamic acid.

temperature between 6 K and 80 K. The addition of a hydroxamic acid, even in great excess, did not cause the copper to become EPR "visible".

The effect of hydroxamic acids upon the "browning" response of whole tissue slices

It is well known that the "browning" response observed when a wide variety of plant materials are cut is caused by tyrosinase activity of the tissue [23]. Use was made of this to test the effect of the hydroxamic acids upon the tyrosinase activity of whole tissue slices. It was found that the hydroxamic acids had dramatic inhibitory effects upon the browning response of potato and apple slices. In this experiment, tissue slices were cut and immediately soaked for 10 min in a solution of inhibitor at the required concentration. Excess water was removed from the slices and they were left to age in air for several hours. For all three hydroxamic acids, a concentration of 10 μM inhibitor was found to be sufficient to completely inhibit the browning responses of both tissues.

The effects of hydroxamic acids on horseradish peroxidase

Schonbaum [9] has already demonstrated that a complex is formed between benzhydroxamic acid and horseradish peroxidase. He further demonstrated that the effect was competitive with the reducing substrate. On the basis of spectral, EPR and kinetic data, he concluded that the binding was by polyfunctional hydrogen bonding.

We studied the effects of 0.5 mM salicylhydroxamic acid on the EPR-detectable forms of horseradish peroxidase. At low (6 K) temperatures, a number of high spin (5/2) and mixed spin (3/2 cf. ref. 24) forms which are in pH-depen-

dent equilibrium can be observed. Addition of the hydroxamic acid caused conversion into a single, pH-independent, rhombic, high spin heme configuration (Fig. 4). The number of detectable spins in this form increases several-fold because the low spin forms (not detectable because of saturation at this temperature) are also converted into this configuration and so become EPR detectable. This effect is directly analogous to that reported by Leigh et al. [25] for the binding of a typical substrate, hydroquinone, to the horseradish peroxidase. The binding in both cases is presumably close enough to the heme group to cause conformational restraints which favor the single high spin heme configuration (cf. ref. 25).

Since the hydroxamic acids are able to chelate ferric iron much more efficiently than ferrous iron [4,26], one would expect the midpoint potential of

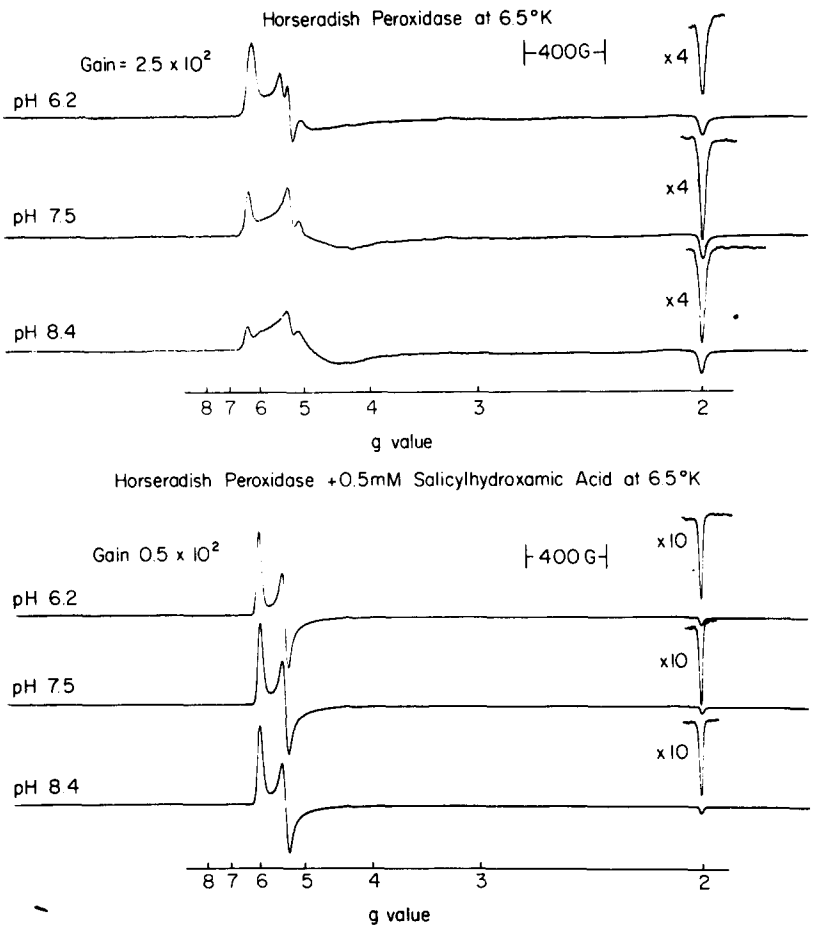


Fig. 4. EPR-detectable effects of salicylhydroxamic acid on horseradish peroxidase. Horseradish peroxidase (Sigma, grade VI) was dissolved in an appropriate buffer to 5 mg/ml. Buffers used were: pH 6.2, 50 mM, 2-(*N*-morpholino)-ethane sulfonate; pH 7.5, 50 mM methyl-2-(*N*-tris(hydroxymethyl) aminoethane) sulfonate; pH 8.4, 50 mM glycylglycine. Samples with or without 0.5 mM salicylhydroxamic acid were frozen rapidly in liquid nitrogen and stored at 77 K until assayed. Conditions of EPR measurement were: modulation aplitude, 12.5 G; modulation frequency, 100 kHz, microwave power, 5 mW; microwave frequency, 9.185 GHz; temperature, 6.5 K.

the heme iron of the horseradish peroxidase to be significantly shifted to lower potentials on addition of a hydroxamic acid if metal chelation were indeed the mechanism of inhibition. The shift would be —60 mV per 10-fold difference in affinity for oxidized compared to reduced forms (see Clark [27] for a detailed mathematical treatment). Such a titration carried out anaerobically in the presence of redox mediators as discussed by Dutton [18] is illustrated in Fig. 5. The pH dependency of the midpoint potential obtained with the native enzyme is essentially the same as that obtained by Harbury [28]. A slope of —60 mV/pH is found, until the pK of 7.3 of the H^+ is reached when the redox reaction becomes pH independent. It can be seen that the addition of a hydroxamic acid at pH 7.0 caused at most a 100 mV negative shift in the $E_{m\,7}$ of —250 mV of the peroxidase heme. A slope of —60 mV/pH unit is still observed in the salicylhydroxamic acid-complexed enzyme, again with a pK of around 7.3. Considering that the affinity of hydroxamic acids is many orders of magnitude higher for ferric, compared to ferrous, iron [4,26], this E_m change is much less that one would expect if indeed the iron of the enzyme were being directly chelated by the inhibitor. It does still, however, indicate a preference of the hydroxamic acid for the oxidized form of the enzyme as compared to the reduced form by almost two orders of magnitude. We would attribute this to a conformational effect of the heme reduction on the substrate binding site, which converts the site into a configuration less favorable to hydroxamate binding. Only in this way can we still rationalize the kinetic [9] and EPR effects of the hydroxamate.

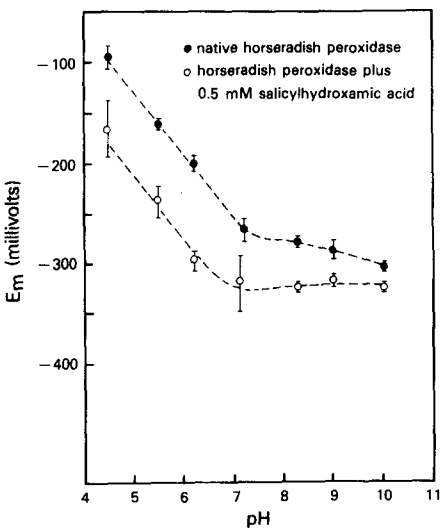


Fig. 5. Redox titration of horseradish peroxidase in the absence and presence of salicylhydroxamic acid. A solution of 0.25 mg/ml horseradish peroxidase in appropriate buffer was made anaerobic with a flow of argon gas. Mediators added were benzyl viologen (10 μM), indigo-2-sulfonate (10 μM), 2-hydroxy-1,4-naphthoquinone (10 μM), anthraquinone-2,6-sulfonate (10 μM) and phenosafranine (5 μM). A spectrophotometric redox titration was performed as described by Dutton [18] with wavelength of 435 minus 450 nm. Sodium dithionite and potassium ferricyanide were used for the reductive and oxidative titrations, respectively, and care was taken that hysteresis did not occur, i.e. that the system was in equlibrium. Buffers used were: pH 4.5—5.5, 50 mM succinic acid; pH 6.2, 50 mM 2-(*N*-morpholino)-ethane sulfonate; pH 7—7.5, 50 mM potassium phosphate; pH 8.5—10.5, 50 mM glycylglycine.

Discussion

It is clear that hydroxamic acids, originally reported to be specific inhibitors of the higher plant mitochondrial alternative oxidase [18], are rather less specific inhibitors than once expected. This has given us a means of studying their mode of inhibition directly by testing their effects on well characterized redox enzymes. The accumulated results obtained with both mushroom tyrosinase and horseradish peroxidase indicate that a mechanism of inhibition whereby the hydroxamic acid chelates the metal from the active site of the enzyme is not the most likely mechanism, even although hydroxamic acids have previously been noted for their metal-chelating capacity. This evidence includes the reversible nature of inhibition, competitive kinetics with respect to reducing substrate, lack of correlation of inhibition with metal prosthetic group type, EPR perturbation effects, and thermodynamic measurements. Further, recent studies in collaboration with workers in the laboratory of Dr. A. Cerami, Rockefeller University, New York, have shown that the potential of inhibition of a series of hydroxamic acid derivatives is dependent not upon their metal-chelating ability, but instead upon the presence of an aryl moiety on a primary hydroxamate group (unpublished data).

From the experiments performed, we feel that an alternative reasonable explanation of hydroxamic acid inhibition is that of competition with the phenolic substrate for its binding site on the protein. This binding, by conformational changes of the protein, presumably may also affect the kinetics of the enzyme with respect to oxygen binding at the metal (cf. tyrosinase inhibition kinetics) or can cause conformational restraints on the number of possible heme configurations (cf. the EPR-detectable effects of salicylhydroxamic acid on horseradish peroxidase), if the substrate binding site is close enough to the heme group. The binding, which is easily reversible, may be by polyfunctional hydrogen bonding (cf. ref. 1), as suggested originally by Schonbaum [9]. However, it is also known that hydroxamic acids can undergo a 1-electron oxidation in the presence of acidic ceric sulfate or alkaline ferricyanide to give a short-lived radical intermediate [29]. It is thought extremely unlikely that the hydroxamic acids are actually oxidized to such products when they act as inhibitors in these systems, for example, no transient oxygen consumption could be detected on addition of hydroxamic acids to tyrosinase, and no radical species were detected in any inhibitor · enzyme complexes (unpublished observations of Rich, P.R. and Bonner, Jr., W.D.). Instead, however, it is quite feasible that a partial electron sharing may occur between the hydroxamic acid and an acceptor group in the enzyme to produce a "charge transfer complex" [30,31]. Such a hypothesis is experimentally testable, since a charge transfer absorption band should be detectable with a wavelength maximum dependent upon the ionisation potential of the hydroxamic acid species. Experiments designed to detect such absorption bands are currently in progress.

These conclusions may be extended to another hydroxamic acid-inhibitable system, that of the alternative mitochondrial respiratory pathway of higher plants [8]. We have already demonstrated that the commonly accepted explanation of inhibition of the alternative respiratory oxidase by metal chelation, presumably of the iron in an iron-sulfur center, is not borne out by experimen-

tal findings [32—34]. These new results offer an alternative explanation, that of competition with respect to the reducing substrate of the terminal oxidase, which in this case would be a reduced form of ubiquinone [35]. A mechanism of this kind would explain the observed anomalies that the K_i of inhibition is dependent upon electron flux [36] and that the salicylhydroxamic acid inhibitory effect is not competitive with respect to oxygen concentration (Rich, P.R., unpublished data).

Since the hydroxamic acids may actually have multiple effects upon major biochemical processes in the plant, an effect of these chemicals observed in an unpurified or whole cell system is not easy to interpret. The observation is particularly relevant to the effects of these hydroxamic acids upon tissue respiration, where in several instances a reduction in oxygen consumption on addition of a hydroxamic acid has been taken as evidence of an alternative mitochondrial respiratory pathway. The work reported in this paper suggests that an assumption of this nature is not justifiable. For example, the total tyrosinase of a potato slice has at least 100 times the oxygen-consuming capacity of the respiratory pathway and is inhibited by at least one order of magnitude less by hydroxamic acid than is the alternative mitochondrial pathway.

Acknowledgments

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