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Specific Inhibition of the Cyanide-insensitive Respiratory Pathway in Plant Mitochondria by Hydroxamic Acids

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ABSTRACT

Hydroxamic acids, R-CONHOH, are inhibitors specific to the respiratory pathway through the alternate, cyanide-insensitive terminal oxidase of plant mitochondria. The nature of the R group in these compounds affects the concentration at which the hydroxamic acids are effective, but it appears that all hydroxamic acids inhibit if high enough concentrations are used. The benzhydroxamic acids are effective at relatively low concentrations; of these, the most effective are *m*-chlorobenzhydroxamic acid and *m*-iodobenzhydroxamic acid. The concentrations required for half-maximal inhibition of the alternate oxidase pathway in mung bean (*Phaseolus aureus*) mitochondria are 0.03 mM for *m*-chlorobenzhydroxamic acid and 0.02 mM for *m*-iodobenzhydroxamic acid. With skunk cabbage (*Symplocarpus foetidus*) mitochondria, the required concentrations are 0.16 for *m*-chlorobenzhydroxamic acid and 0.05 for *m*-iodobenzhydroxamic acid. At concentrations which inhibit completely the alternate oxidase pathway, these two compounds have no discernible effect on either the respiratory pathway through cytochrome oxidase, or on the energy coupling reactions of these mitochondria. These inhibitors make it possible to isolate the two respiratory pathways and study their mode of action separately. These inhibitors also enhance an electron paramagnetic resonance signal near $g = 2$ in anaerobic, submitochondrial particles from skunk cabbage, which appears to be specific to the alternate oxidase and thus provides a means for its assay.

Mitochondria isolated from a number of plant tissues show incomplete inhibition of respiration by cyanide. Outstanding in this respect are mitochondria isolated from the spadices of aroids; in particular, *Arum maculatum* (1, 4) and skunk cabbage, *Symplocarpus foetidus* (2, 12, 13, 31), which show little, if any, sensitivity to cyanide inhibition. Mitochondria from the hypocotyls of etiolated mung beans (*Phaseolus aureus*) show partial sensitivity; approximately 70% of the state 3 rate is inhibited by cyanide or antimycin A (16). In contrast, the respiration of mitochondria isolated from potato tubers (*Solanum tuberosum*) shows nearly complete inhibition by either of these compounds. Bendall and Bonner (2) have critically evaluated the various hypotheses which have been proposed to explain this behavior and conclude from their results and those of Storey and Bahr (24) that it is best explained by a branched electron transport pathway from substrate to oxygen through cyanide-sensitive cytochrome oxidase and a cyanide-insensitive alternate oxidase (5). Location of the alternate oxidase in the flavoprotein region of the plant respiratory chain was first reported by Bendall *et al.* (3). This location, and the possible nature of the alternate oxidase, have been discussed in detail by Bendall and Bonner (2). They found no absorbance spectrum or other physical property specific to the oxidase, even in the presence of compounds—in particular, KSCN—which inhibit the respiratory pathway through the alternate oxidase. One difficulty was that inhibition was observed only at concentrations of these compounds so high that unwanted secondary reactions could occur.

In this paper we report that hydroxamic acids, in particular the benzhydroxamic acids, inhibit the alternate pathway. The inhibitors are completely specific to the alternate pathway and affect neither energy coupling nor electron transport through the cytochrome pathway. Kinetic experiments with skunk cabbage mitochondria (9) have showed that the site of hydroxamic acid inhibition is located between the fluorescent high potential flavoprotein Fp_{hf} (23) and oxygen. The inhibitiors enhance an EPR[2] signal near $g = 2$ in anaerobic submitochondrial particles, and thus open the possibility of a direct assay for the alternate oxidase.

MATERIALS AND METHODS

Mitochondria were prepared from the excised hypocotyls of six-day-old etiolated mung bean (*Phaseolus aureus*) seedlings and fresh white potato tubers, following the methods of Bonner (7) and Ikuma and Bonner (15). Mitochondria from the spadices of skunk cabbage flowers (*Symplocarpus foetidus*) were prepared as described previously (24).

The respiratory activity of each mitochondrial preparation was determined polarigraphically with a Clark electrode (Yellow Springs Instrument Co.) as described by Estabrook (10). The reaction medium was either medium A containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 5 mM phosphate, at pH 7.2, or medium TP containing 0.3 M mannitol, 10 mM tris(hydroxymethyl)methylaminoethyl sulfonic acid, and 5 mM phosphate, at pH 7.2. There is no difference in mitochondrial behavior in these two media. All commercially available reagents were of the highest grade and were used without further purification. The uncoupler 1799 was generously provided by Dr. P. Heytler of E. I. duPont de Nemours & Co., Inc. The benzhydroxamic acids were syn-

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[2] Abbreviations: EPR: electron paramagnetic resonance; mCLAM: *m*-chlorobenzhydroxamic acid; mIBM: *m*-iodobenzhydroxamic acid; 1799: *bis*-(hexafluoroacetonyl)acetone; SHAM: salicylhydroxamic acid.

thesized by established procedures (14). Stock solutions of each compound were prepared in dimethylformamide or in ethanol. Submitochondrial particles for EPR studies were prepared from skunk cabbage mitochondria by the osmotic shock method of Wilson and Bonner (30). EPR spectra were obtained on a Varian H502 spectrometer.

RESULTS

An oxygen electrode record showing the effect of the sequential addition of ADP, KCN, and mCLAM on coupled mung bean mitochondria oxidizing succinate is presented in Figure 1A. In accord with the findings of Ikuma and Bonner (16), the mitochondria were pretreated aerobically with ATP in order to achieve the state 4 rate with this substrate. An ADP phosphorylation cycle with a respiratory control ratio of 1.7 and an ADP–O ratio of 1.6 (8) ensues upon addition of 130 μM ADP. Addition of 0.27 mM KCN reduces the respiratory rate to 25% of that in state 3; subsequent mCLAM addition reduces the respiratory rate to zero. Figure 1B presents evidence that mCLAM is completely specific to the cyanide-insensitive pathway. In this experiment, mCLAM is added to the mitochondrial suspension prior to succinate. Subsequent addition of ADP produces a phosphorylation cycle with the respiratory control and ADP-O ratios both near 2.0. The mitochondria pretreated with mCLAM are now completely sensitive to cyanide inhibition, since 0.27 mM KCN effectively stops respiration. The inhibitor appears to improve both the respiratory control and the ADP-O ratios with succinate as substrate, presumably by blocking the nonphosphorylating pathway through the alternate oxidase.

This effect is strikingly illustrated with skunk cabbage mitochondria, as shown by the oxygen electrode records in Figure 2. The mitochondria are treated with ATP to achieve the state 4 rate with succinate as substrate (16). Sequential addition of KCN, the uncoupler 1799, and mCLAM to the mitochondrial suspension respiring with succinate produces a 14% inhibition of the rate by cyanide, no further stimulation by 1799, and 90% inhibition by mCLAM (Fig. 2A). Characteristically, 10% or less

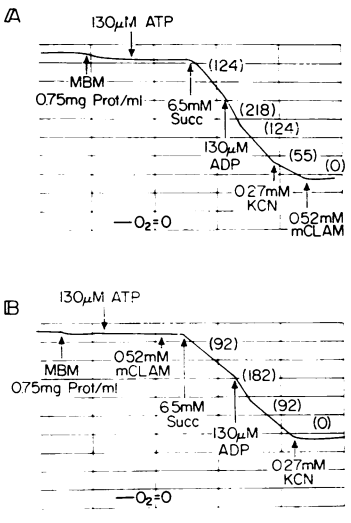

FIG. 1. A: Recording obtained of oxygen consumption measured with the Clark electrode at 24 C by mung bean mitochondria (MBM) suspended in medium TP with succinate as substrate, showing partial insensitivity to respiratory inhibition by KCN added in State 4. Subsequent addition of mCLAM reduces the respiratory rate to nil. B: Recording obtained under the same conditions as in A, but with mCLAM added prior to addition of succinate. The respiration is now completely sensitive to the addition of KCN at the point indicated.

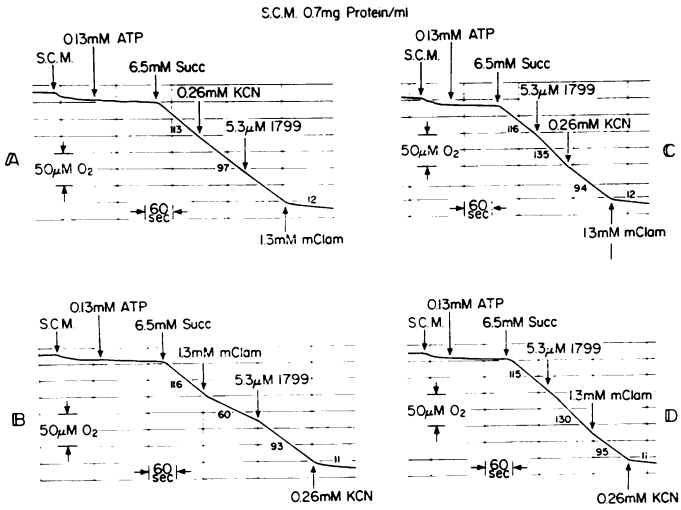

FIG. 2. Recordings of oxygen consumption measured with the Clark electrode at 24 C of skunk cabbage mitochondria (SCM) suspended in medium TP with succinate as substrate. A: Addition of mCLAM after KCN and 1799, showing inhibition of the cyanide-insensitive respiration. B: Addition of mCLAM before 1799 and KCN, showing state 4 rate through the coupled cytochrome oxidase pathway. C: Addition of 1799 before the respiratory inhibitors, showing slight stimulation by the uncoupler. D: Addition of mCLAM after 1799, showing that this inhibitor does not inhibit the uncoupled cytochrome oxidase pathway.

residual respiration is observed with skunk cabbage mitochondria after saturating concentrations of KCN and mCLAM have been added. The record shown in Figure 2B was obtained by reversing the sequence of mCLAM and KCN addition. Addition of mCLAM inhibits the original respiratory rate with succinate by 45%, but subsequent addition of uncoupler restores the rate of 85% of its original value. Respiration is then nearly stopped by KCN. The rate observed with mCLAM is the state 4 rate through the cytochrome pathway alone, while that with mCLAM and 1799 is the state 3 rate. The respiratory control ratio calculated from these two rates is 1.5. In the absence of mCLAM, this ratio drops to 1.2, as shown in the experiment recorded in Figure 2C, in which uncoupler was added prior to the respiratory inhibitors in order to obtain the maximum state 3 rate directly from state 4. The addition sequence of mCLAM and KCN are reversed in the experiment of Figure 2D, which shows that mCLAM reduces the respiratory rate to that expected for the cytochrome pathway alone, but otherwise has no direct inhibitory effect on succinate oxidation. It is evident from the experiments shown in Figure 2 that the capacities of both the cytochrome oxidase and alternate pathways are nearly equal, and each one alone is capable of carrying nearly the full respiratory flux.

Since potato mitochondria have little cyanide-insensitive respiration, they are especially suited for an examination of the effects of hydroxamic acids on the normal respiratory chain. The experiments were conducted in the same manner as those in Figure 1B, with mIBM. Figure 3A shows that the state 3 rate is unaffected by mIBM except at the highest concentrations, while the state 4 rate increases somewhat with increasing mIBM concentration, thereby decreasing the respiratory control ratio (Figure 3B). This slight uncoupling activity does not lead, however, to any substantial effect on the ADP-O ratio (Fig. 3B). Thus, in interpreting the effects of hydroxamic acids on cyanide-insensitive mitochondria, one may neglect their effects on the normal respiratory pathway.

Twelve hydroxamic acids were examined as inhibitors of the

respiratory pathway through the alternate oxidase. The oxygen consumption rates of mitochondria from both mung bean hypocotyls and skunk cabbage spadices in state 4, with succinate as substrate in the presence of 0.3 mM KCN, were titrated with these inhibitors, and values of the concentration required for half-maximal inhibition were determined. These values are collected in Table I. It is apparent from Table I that: (*a*) all the hydroxamic acids inhibit the alternate pathway, but the amounts required for mitochondria from a given source vary by a factor of approximately seven; (*b*) skunk cabbage mitochondria require an inhibitor concentration four to six times higher for half-maximal inhibition than do mung bean mitochondria.

With mCLAM and SHAM the inhibition titer was independent of the mitochondrial protein concentration in the reaction me-

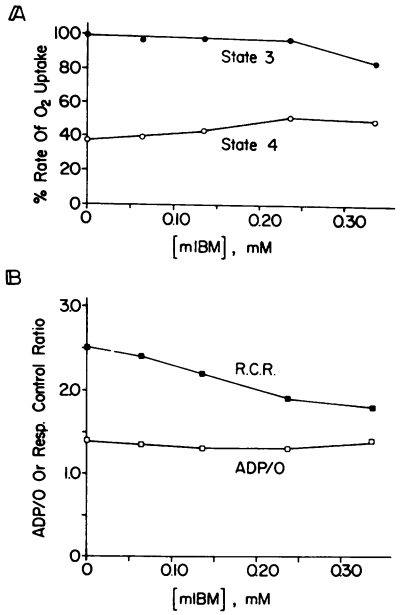

FIG. 3. Effect of mIBM concentration on oxygen uptake in state 3 and state 4 (A) and on the respiratory control ratio (R.C.R.) and ADP/O ratio (B) of white potato mitochondria. The mitochondria were suspended in medium A with 7 mM succinate as substrate.

Table I. *Concentrations of Hydroxamic Acid for Half-maximal Inhibition of the Respiratory Rate of Mitochondria in State 4*

Succinate 6.5 mM was utilized as substrate in the presence of 0.3 mM KCN.

Hydroxamic Acid	Inhibitor Concn	
	Skunk cabbage	Mung bean
	mM	
Cyclohexyl-	1.30	0.18
Phenylacet-	0.89	0.16
Isonicotinic-	0.81	0.24
Benz-	0.40	0.09
m-Toluo-	0.35	0.09
m-Fluorobenz-	0.24	0.06
m-Nitrobenz-	0.30	0.06
m-Chlorobenz-	0.16	0.03
m-Iodobenz-	0.05	0.02
p-Chlorobenz	0.32	0.09
o-Carboxybenz-	1.07	0.21
Salicyl-	0.26	0.06
2-Naphthyl-	0.22	

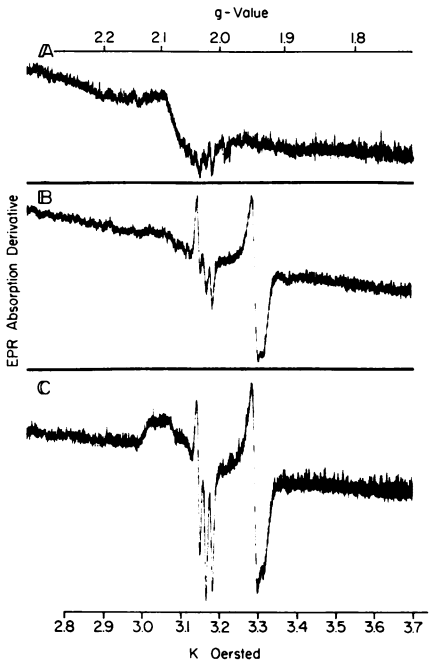

FIG. 4. EPR spectra of submitochondrial particles from skunk cabbage spadix obtained at 77 K. Instrument settings: modulation amplitude, 12 gauss; microwave frequency, 8.98 GHz. A: Aerobic particles; B: particles made anaerobic with NADH as substrate; C: particles made anaerobic with NADH in the presence of saturating mIBM.

dium over a range of 0.2 to 0.9 mg protein/ml. The inhibitor mCLAM was readily removed from skunk cabbage mitochondria by washing with 0.3 M mannitol medium, thereby restoring cyanide-insensitive respiration. These results demonstrate that the hydroxamic acid inhibitors are freely dissociable from the mitochondria, and that they do not irreversibly damage the components of the alternate oxidase pathway.

Attempts to obtain an optical absorbance difference spectrum characteristic of the alternate oxidase by means of these inhibitors were as unsuccessful as those of Bendall and Bonner (2). On the other hand, a characteristic EPR spectrum was obtained as shown in Figure 4 for skunk cabbage submitochondrial particles in the presence and absence of mIBM. The small signal seen with aerobic particles is shown in Figure 4A. Addition of mIBM to the aerobic particles gives the same EPR spectrum. In anaerobic particles, however, the EPR spectrum is well defined with components near $g = 2$ and $g = 1.94$, whose intensity is further enhanced by mIBM, particularly in the region near g = (2 Fig. 4C). The enhanced EPR signal induced by mIBM is best attributed to a previously unidentified component in the alternate respiratory pathway, very likely the oxidase itself. The complexity of the signal does not permit us to draw any conclusions concerning the nature of this component at present. The existence of the enhanced signal does provide, for the first time, a specific assay for those components which comprise the alternate oxidase and thus opens the way to isolation and characterization of the oxidase itself.

DISCUSSION

Structural Requirements for Inhibition. The hydroxamic acids are reversible, specific inhibitors of the cyanide-insensitive respiratory pathway of skunk cabbage and mung bean mitochondria and appear to have no effect on the pathway involving the cytochromes. It appears probable from Table I that all hydroxamic

acids are capable of acting as inhibitors of the alternate oxidase if sufficiently high concentrations are used. The hydroxamic acid group itself, —CONHOH, is evidently the functional group required for the inhibitory effect.

Concentrations of R—CONHOH which are required for 50% inhibition of the mitochondrial respiratory rate through the alternate pathway fall within the relatively narrow limits of 0.70 ± 0.50 mM for skunk cabbage and 0.12 ± 0.10 mM for mung beans. Such behavior is particularly remarkable if one considers the significant physico-chemical differences among the compounds listed in Table I, including differing solubility in polar solvents, different steric requirements, and altered electronic effects transmitted to the carbonyl group. While such parameters dominate the interaction of hydroxamic acids with various plant peroxidases (22), their influence on the inhibition of the cyanide-insenstive oxidase can only be of secondary importance. This may indicate that the oxidase reaction site is not defined by overly rigid steric requirements. The greater potency of the benzhydroxamic acids, particularly those with halogen substituents, suggest further that the reaction site is located in a nonpolar environment.

Possible Mechanisms of Inhibition. The chemical reactions in which the —CONHOH group may participate should provide a clue concerning the mechanism of inhibition, and this in turn should provide some insight into the nature of the alternate oxidase. These reactions are the following: (*a*) complexation with a Lewis acid, (*b*) polyfunctional H-bonding, (*c*) nucleophilic substitution, (*d*) redox reactions. The redox reactions of hydroxamic acids are probably not relevant in this instance; the acids are not reduced or oxidized on the time scale of the oxygen electrode experiments. Otherwise the inhibitory effect would be transient, which is not observed. It is doubtful that the nucleophilic reactivity of hydroxamic acids can be responsible for the observed inhibition. A stable, irreversibly bound derivative cannot be formed since full oxidase activity is restored following removal of the hydroxamic acid by washing. The lack of any discernible effect on oxidative phosphorylation further lessens the likelihood that hydroxamate nucleophilicity plays a role in the inhibitory mechanism. In this regard, hydroxamic acids behave unlike hydroxylamine; the latter inhibits ATP formation (27, 28, 29) although both share similar reactivities in a variety of nucleophilic substitution reactions (11, 17).

One plausible mechanism of inhibition is complex formation by chelation of a transition metal ion. Among such derivatives, the hydroxamic acid complexes with ferric ion are best known (19) but by no means unique; copper, manganese, and cobalt also form chelates albeit with a far lower affinity than that which is characteristic of Fe^{3+}. This mechanism does require, however, that the stability constant for metal-hydroxamate complex formation in the alternate oxidase be several orders of magnitude greater than that of the corresponding cyanide complex. Although such a difference in favor of the hydroxamates as ligands seems most unlikely, it cannot be entirely discounted.

An alternative to metal binding is the possibility that polyfunctional hydrogen bonding is responsible for the inhibitory effect of hydroxamic acids. This interpretation is analogous to the proposals advanced as explanations of the peroxidase-hydroxamate interaction (22) and emphasizes a structural feature which, although common to β-keto-enol systems, is expressed particularly strongly by the —CONHOH group. Polyfunctional hydrogen bonding might stabilize one geometry of ligand binding to a transition metal complex at the active site of the oxidase or prevent a conformational change of the alternate oxidase required for electron transport to oxygen. The EPR signal observed in anaerobic submitochondrial particles from skunk cabbage on addition of mIBM (Fig. 4C) might be interpreted, albeit with caution, as evidence for the latter alternative. No drastically new EPR signature is elicited on addition of mIBM to the anaerobic particles, as would be expected if complexation of an active site metal ion with the hydroxamic acid actually occurred. Rather, the signals around $g = 2$ are markedly enhanced and the signal shape is somewhat altered, suggesting that the alternate oxidase may exist in two conformations, one which is inactive in electron transport, is stabilized by the hydroxamic acid, and gives a stronger EPR signal at $g = 2$. Further insight into the nature of the alternate oxidase and the mechanism of its inhibition by hydroxamic acids must await future experimental results.

Location of the Alternate Oxidase. Hydroxamic acids are known to be good nucleophiles in substitution reactions at phosphorus (11). Yet the phosphorylation of ADP to ATP, a reaction in which inhibition would be expected, is completely unaffected by hydroxamic acids in these mitochondria. The simplest hypothesis is that the inner mitochondrial membrane is impermeable to the hydroxamic acids; they do not reach the ATPase mediating the phosphorylation which is on the inside of the inner membrane in intact mitochondria (18, 21, 26). This hypothesis in turn requires that the alternate oxidase be located on the outside of the inner membrane, in which location it would be freely accessible to the hydroxamic acid inhibitors through the highly permeable outer mitochondrial membrane (6, 20). The hypothesis should be testable; it implies that, like cytochrome *c*, the alternate oxidase should be extractable directly from intact mitochondria. An outside location for the alternate oxidase suggests that the plant cell can control the electron transport capacity of the alternate pathway of its mitochondria by regulating directly the synthesis of the alternate oxidase.

The Alternate Pathway and Respiratory Control. Inhibition of the alternate pathway in skunk cabbage mitochondria by mCLAM increases the respiratory control ratio (Fig. 2) indicating that the alternate pathway makes a substantial contribution to the total flux in state 4. The ADP-O ratios reported previously (25) for phosphorylating skunk cabbage mitochondria led to the conclusion that the contribution of the alternate pathway to the flux in state 3 is small. In skunk cabbage mitochondria, the maximal electron transport capacity of the alternate pathway is about equal to that of the cytochrome pathway, and each one is capable of handling nearly the total flux made available by the substrate dehydrogenases. In mung bean mitochondria, however, the maximal capacity of the alternate pathway is less than the state 4 rate with succinate. Its contribution to the flux in state 3 and state 4 with succinate in these mitochondria appears fairly constant from a comparison of Figure 2, A and B. In the absence of mCLAM, the state 4 rate is faster by an increment of 32 natoms O/min·mg protein, whereas the state 3 rate is faster by 34 natoms O/min·mg protein. A detailed quantitative study of the relative contributions of the alternate and cytochrome respiratory pathways in different mitochondria will be reported in a future publication. It suffices to point out here that thermogenic tissues such as the skunk cabbage spadix and nonthermogenic tissues such as the mung bean hypocotyl appear to use the same alternate oxidase pathway, and that the differences between the two are quantitative differences in activity rather than differences in mechanism.

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