The Reaction of the Soybean Cotyledon Mitochondrial Cyanide-resistant Oxidase with Sulfhydryl Reagents Suggests That α-Keto Acid Activation Involves the Formation of a Thiohemiacetal*

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The cyanide-resistant alternative oxidase of plant mitochondria is known to be activated by α-keto acids, such as pyruvate, and by the reduction of a disulfide bond that bridges the two subunits of the enzyme homodimer. When the regulatory cysteines are oxidized, the inactivated enzyme is much less responsive to pyruvate than when these groups are reduced. When soybean cotyledon mitochondria were isolated in the presence of iodoacetate or N-ethylmaleimide, the intermolecular disulfide bond did not form and the alternative oxidase was present only as a noncovalently associated dimer. N-Ethylmaleimide inhibited alternative oxidase activity, but iodoacetate was found to stimulate activity much like pyruvate, including enhancing the enzyme’s apparent affinity for reduced ubiquinone. The presence of pyruvate or iodoacetate blocked inhibition of the enzyme by N-ethylmaleimide, indicating that all three compounds acted at the same sulfhydryl group on the alternative oxidase protein. The site of pyruvate and iodoacetate action was shown to be a different sulfhydryl than that involved in the redox-active regulatory disulfide bond, because iodoacetate bound to the alternative oxidase at the activating site even when the redox-active regulatory sulfhydryls were oxidized. Given the nature of the covalent adduct formed by the reaction of iodoacetate with sulfhydryls, the activation of the alternative oxidase by α-keto acids appears to involve the formation of a thiohemiacetal.

Plant mitochondria possess a terminal cyanide-resistant alternative oxidase in the electron transport system of the inner membrane (1, 2). This oxidase reduces oxygen to water with electrons derived directly from the ubiquinone pool. Because no protonotive force is generated during this reaction, the alternative pathway appears to be energetically wasteful (1, 2), cDNA sequences have now been reported for the nuclear-en
coded oxidase from several plants, two fungi, and a protozoon (2–7).1 The mature protein contains 280–290 amino acids and migrates as a 32–35-kDa protein on SDS-PAGE gels as revealed by immunoblotting. Hydrophathy analysis has led to a model in which the alternative oxidase protein is anchored to the mitochondrial inner membrane by two membrane-spanning α-helices, with large hydrophilic domains approximately 100 amino acids in length flanking each membrane-spanning region and extending into the mitochondrial matrix (9). In the carboxyl-terminal hydrophilic domain, just beyond the second membrane-spanning region, is a set of conserved amino acid motifs found in the family of coupled binuclear iron proteins to which methane monooxygenase belongs (9, 10). Cross-linking studies have indicated that the plant alternative oxidase exists in the membrane as a homodimer (11).

As a result of a search for the role of the alternative oxidase in plant metabolism, several regulators of its activity have been identified. The amounts of alternative oxidase protein in the membrane (e.g. Refs. 12–14) and the extent of ubiquinone pool reduction (e.g. Ref. 15) are well established as exerting control over alternative oxidase activity. The concentration of ubiquinone in mitochondria has been shown to vary among tissues and is likely also to act as a parameter regulating alternative oxidase activity (16). Two additional types of regulation of alternative oxidase activity are also known. One of these arises from a pair of redox-active sulfhydryls which, when oxidized, covalently cross-link the two subunits of the alternative oxidase homodimer (11). In the oxidized state, the enzyme is much less active than when the bond is reduced to its constituent sulfhydryls. The other regulatory feature is the reversible stimulation of alternative oxidase activity by α-keto acids (2, 17). Pyruvate has been the most studied α-keto acid (18–22), and it stimulates alternative oxidase activity by decreasing the enzyme’s apparent $K_m$ for reduced ubiquinone (19, 22), although an increase in the $V_{max}$ may also accrue (23). This effect is probably due to the direct interaction of pyruvate with a domain of the protein on the matrix side of the inner membrane (17, 20), although the exact site of pyruvate action is unknown.

Pyruvate has little effect on alternative oxidase activity when the monomeric subunits are covalently linked by the redox-active regulatory disulfide bond (19). Only when this disulfide bond is reduced can maximal stimulation with pyruvate be achieved. Thus, the reduced state of the regulatory sulfhydryl/disulfide is required to allow the pyruvate-induced change that results in enhanced activity. Determination of the site of pyruvate action and its relationship to the redox-active regulatory sulfhydryls would lead to a better understanding of how these two factors affect the structure of the enzyme to bring about its regulation. We have used sulfhydryl reagents to examine this relationship and further define the role of sulfhydryl groups in the regulation of alternative oxidase activity.

EXPERIMENTAL PROCEDURES

Plant Material—Soybean seedlings (Glycine max (L.) Merr. cv Ransom, Essex, or FF583) were grown in a greenhouse as described previously (11). Tissue was harvested between 6 and 15 days after planting, depending on the season of the year.

Isolation of Mitochondria—Mitochondria were isolated on Percoll gradients from soybean seedling cotyledons according to Umbach and

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1 Alternative oxidase of Trypanosoma brucei brucei, GenBank™ accession number U52964.

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The concentration of 10 mM in the presence of 150 mM ampicillin. When succinate was used as substrate, it was at a final concentration of 2 mM in the wash buffer and in the Percoll gradient. In all cases, the final mitochondrial wash and resuspension was done with wash buffer. NEM was added from a fresh 2M sulfoxide stock solution. Mitochondria were treated with 3 mM iodoacetate before SDS-PAGE. Note: there are three molecular mass species of oxidized dimers in the 60–70-kDa range, apparently due to the mixed association of the two monomeric species present in soybean cotyledon mitochondria (11).

**Alternative Oxidase Activity Measurements**—Oxygen consumption was measured with a Clark-type oxygen electrode in a 1.8-ml reaction chamber. All measurements were conducted at 25°C in a reaction mixture that consisted of 10 mM potassium phosphate, pH 7.0, 10 mM KCl, 5 mM MgCl₂, 0.3 mM mannitol, and 0.1% (w/v) BSA. When duroquinol was the substrate, 1 mM EDTA was included. Additions made during the measurements were (final concentration given): mitochondrial at 0.1–0.2 mg ml⁻¹ protein, 1.0 mM NADH or 0.5 mM duroquinol, 50 μM ADP, 6 μM myoxihialo, 5 mM pyruvate, and 2 mM salicylhydroxamic acid. When succinate was used as substrate, it was at a final concentration of 10 mM in the presence of 150 μM ATP. Unless stated otherwise, one or two state 3/4 transitions were induced with ADP before addition of myoxihialo, and all rates reported are after substraction of any residual rate measured in the presence of myoxihialo and salicylhydroxamic acid. When used during the course of activity assays, sulphydryl reagents were added to a final concentration of 5 mM from stocks made on the day of the experiment. Iodoacetate was prepared in reaction buffer at 400 mM, and NEM was prepared at 1M in dimethyl sulfoxide. For experiments in which the effects of diamide and DTT on alternative oxidase activity were determined, mitochondria isolated with or without iodoacetate were treated with these reagents (3 and 20 mM final concentrations, respectively) and washed twice in excess volume of wash buffer before any subsequent assays or procedures, as described previously (11).

**Voltammetric Determination of the Reduction Level of the Mitochondrial Ubiquinone Pool**—The reduction state of the mitochondrial ubiquinone pool was measured using a voltammetric apparatus and method based on that described by Moore et al. (25), but with NADH as substrate (26). The reaction medium (2.2 ml) consisted of 10 mM MOPS-KOH, pH 7.4, 0.3 mM mannitol, 10 mM KCl, 1 mM MgCl₂, 10 mM KH₂PO₄, and 1 mM EGTA. To begin the assay, 2 μl ubiquinone-1 was added to the chamber followed by 1 mM NADH from a stock containing 100 mM NADH, 0.3 mM mannitol, and 20 mM MOPS, pH 7.4. At this point, the background deflection caused by NADH was noted and the recorder base line readjusted. Mitochondria (0.15–0.2 mg ml⁻¹ protein) were then added, causing a negligible change in pen deflection, followed by myoxihialo. Because of the presence of 1 mM EGTA, external NADH dehydrogenase activity was initially inhibited, and the ubiquinone pool was initially oxidized. The NADH dehydrogenase activity and the level of ubiquinone pool reduction (Q̄r) were increased by titrating with incremental additions of CaCl₂, added from a stock of 100 mM CaCl₂ in 20 mM MOPS, pH 7.4, up to a final concentration of 2 mM. This titration occurs in the reverse sense from the more common titration through succinate dehydrogenase in which the ubiquinone pool becomes more oxidized as the titration progresses by addition of the succinate dehydrogenase inhibitor malonate (25). For calculations of ubiquinone reduction level, the total reducible ubiquinone pool (Q̄) was taken as the difference between the maximum oxidized level of ubiquinone (after mitochondria were first added) and the maximum reduced level of ubiquinone observed immediately after addition of excess CaCl₂. The total reducible ubiquinone pool size measured with NADH was similar to the pool size measured with succinate as substrate (data not shown). Oxygen uptake rates were monitored simultaneously with ubiquinone reduction level (25).

**SDS-PAGE and Immunoblotting**—Gradient SDS-PAGE gels (10–17%) were run and immunoblotted according to Umbach and Siedow (11). Samples were prepared for the gels in sample buffer from which sulphydryl reductant was omitted. Immunoblots were developed using the alternative oxidase-specific “AOA” antibody (27).

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**References**

1. Moore et al. (25).
2. The abbreviations used are: NEM, N-ethylmaleimide; DTT, dithiothreitol; diamide, azodicarboxylic acid bis(dimethylamide); MOPS, 3-[N-morpholino]propanesulfonic acid; Q, ubiquinone-10; I-Ac, iodoacetate.
Iodoacetate and NEM Bind to the Redox-active Regulatory Sulfhydryls—Two sulfhydryl reagents, iodoacetate and NEM, were used in these experiments. Reaction of iodoacetate with a cysteine sulfhydryl moiety results in the carboxymethylation of the thiol, while reaction with NEM results in maleylation (Fig. 1). When either iodoacetate or NEM was present in the buffers used to isolate mitochondria from soybean cotyledons, no oxidized alternative oxidase dimers were found in the purified mitochondria, whereas mitochondria isolated without the sulfhydryl reagents had pronounced levels of covalently linked dimers (Fig. 2A). In untreated mitochondria, the oxidant diamide markedly enhanced the relative level of covalently cross-linked alternative oxidase dimers, but failed to oxidize the sulfhydryl reagent-reacted alternative oxidase to the covalently linked dimeric form (Fig. 2B), indicating that both sulfhydryl reagents were taken up by the mitochondria during the course of the isolation procedure and had reacted with the sulfhydryl groups involved in the intermolecular disulfide linkage. The sulfhydryl reagent-reacted alternative oxidase could be cross-linked by the homobifunctional lysine-specific cross-linker, ethylene glycolbis(succinimidyl succinate) (data not shown), evidence that the enzyme had maintained its dimeric structure.

Effects of Iodoacetate and NEM on Alternative Oxidase Activity—The alternative oxidase activity of cotyledon mitochondria isolated in the presence of iodoacetate was high with or without pyruvate and was comparable with the alternative oxidase activity of mitochondria isolated without iodoacetate assayed in the presence of pyruvate (Table I). In contrast, mitochondria isolated with NEM showed greatly decreased total respiration and alternative oxidase rates whether or not pyruvate was present (Table I). These assays were conducted using NADH as substrate, because succinate dehydrogenase is not stimulated by pyruvate (Table I). In contrast, the alternative oxidase activity of soybean cotyledon mitochondria isolated with iodoacetate was high with or without pyruvate and was comparable with the alternative oxidase activity of mitochondria isolated without iodoacetate assayed in the presence of pyruvate (Table I). Diamide treatment, which resulted in formation of the intermolecular disulfide bond cross-linking the alternative oxidase subunits (data not shown), depressed alternative oxidase activity of the –I-Ac mitochondria both in the absence and presence of pyruvate (Table I). Interestingly, these diamide-treated mitochondria did display some stimulation when pyruvate was added (Table I). Alternative oxidase activity of the +I-Ac mitochondria, however, was only slightly affected by either DTT or diamide treatment. Furthermore, without pyruvate, the alternative oxidase activity of the +I-Ac mitochondria (whether DTT-treated or not) was as high as that of the DTT-treated –I-Ac mitochondria in the presence of pyruvate (Table I).

These data were suggestive of a common site of action for pyruvate and iodoacetate on the alternative oxidase protein. Data from another type of experiment were consistent with this interpretation. When the sulfhydryl reagents were added directly to respiratory assays of DTT-treated cotyledon mitochondria, they rapidly produced the same effects on alternative oxidase activity as when present during mitochondrial isolation, that is, iodoacetate stimulated and NEM inhibited alternative oxidase activity (Fig. 3). Use of duroquinol as the substrate for these experiments eliminated secondary effects at the external dehydrogenase, although some electron exchange between duroquinol and ubiquinol at the Q site on the cytochrome bc1 complex takes place in this assay (28). When iodoacetate or pyruvate were added sequentially with NEM, the first compound to be added determined the resulting alternative oxidase activity (Fig. 3). Adding pyruvate or iodoacetate before NEM stimulated the alternative oxidase and blocked the inhibitory effects of NEM. Conversely, neither pyruvate nor iodoacetate reversed the inhibition caused by NEM. These results are consistent with a competition for the same site of action by pyruvate, iodoacetate, and NEM.

Iodoacetate, Like Pyruvate, Enhances the Reactivity of the Alternative Oxidase with Reduced Ubiquinone—The interaction of pyruvate and related α-keto acids with the alternative oxidase results in the enzyme becoming active at a lower threshold level of reduced ubiquinone (19, 22). If iodoacetate acts as a true pyruvate analogue, it should affect the alternative oxidase response to reduced ubiquinone in a manner similar to pyruvate. To test this possibility, alternative oxidase activity of soybean cotyledon mitochondria isolated with and without iodoacetate was measured against different levels of reduction of the mitochondrial ubiquinone pool determined voltametrically in reaction mixtures in which external NADH dehydrogenase activity was varied by addition of Ca2+ to an EGTA-containing medium (26). The marked inhibition of suc-
cinate dehydrogenase by iodoacetate precluded our use of succinate as a substrate in these experiments. Similar to results obtained by Hoefnagel and Wiskich using NADH as substrate (23), the alternative oxidase of control mitochondria became active at a lower level of ubiquinone reduction in the presence of pyruvate (Fig. 4). The alternative oxidase in mitochondria isolated with iodoacetate, however, exhibited increased activity at low reduction levels of ubiquinone without added pyruvate (Fig. 4). Furthermore, the presence of pyruvate had no additional effect on the titration of the iodoacetate-complexed alternative oxidase (data not shown). Thus, with respect to the response of the alternative oxidase to ubiquinone pool reduction, iodoacetate substituted for pyruvate.

The Site of Pyruvate and Iodoacetate Action Is Not at the Redox-active Regulatory Sulfhydryl—Because iodoacetate substituted for pyruvate in stimulating alternative oxidase activity and also maintained the enzyme in its reduced form, the possibility existed that the site of pyruvate action might be at the same sulfhydryl group as that involved in the redox-active regulatory disulfide bond. However, this was not the case, as shown by two types of experiments.

In the first type of experiment, during oxygen uptake conditions, DTT-treated cotyledon mitochondria were treated for 5 min with pyruvate, iodoacetate, or NEM after myxothiazol was added. The mitochondria were then removed from the oxygen electrode chamber and, after extensive washing, subjected to oxidation with diamide. Oxidation of the alternative oxidase dimer by diamide occurred in mitochondria assayed with either pyruvate or iodoacetate, as evidenced by the change in the ratio of cross-linked to uncross-linked species (Fig. 5). However, essentially no change in this ratio was seen in NEM-treated mitochondria, suggesting that NEM had blocked subsequent diamide oxidation of the redox-active sulfhydryls (Fig. 5).

To verify that iodoacetate and pyruvate acted at a site distinct from the sulfhydryls involved in the regulatory sulfhydryl/disulfide system, a second type of experiment was performed (Fig. 6A). Mitochondria were first treated with 3 mM diamide to oxidize the intermolecular disulfide bond. Subsequently, the mitochondria (+I-Ac) were added to the oxygen electrode cuvette and substrate ( duroquinol) was added. As oxygen uptake proceeded, iodoacetate was added, replicating the conditions of the previous set of experiments (except that myxothiazol was not present). After 5 min, the mitochondria were removed from the oxygen electrode cuvette, thoroughly washed, and then were treated with DTT to reduce the intermolecular disulfide bond. A second set of mitochondria (−I-Ac) were treated similarly except that iodoacetate was not added during the oxygen electrode assay step. The mitochondria were then assayed for alternative oxidase activity and for the effects of added pyruvate or NEM on that activity. Immunoblots were carried out with mitochondria sampled after iodoacetate addition, to ensure that no disulfide bond reduction had occurred during that period.

![Fig. 3. Response of alternative oxidase activity to sulfhydryl reagents added sequentially during respiratory assays.](image)

![Fig. 4. Response of alternative oxidase activity of soybean cotyledon mitochondria, isolated with and without iodoacetate, to ubiquinone reduction.](image)
Adding pyruvate to these mitochondria had very little effect (Fig. 6C). Thus, pyruvate, iodoacetate, and NEM appear to affect activity by acting at the same sulfhydryl site on the alternative oxidase, and this site is distinct from the sulfhydryls involved in formation of the redox-active regulatory disulfide bond.

Effect of Borohydride on the Alternative Oxidase-pyruvate Association—While iodoacetate is relatively specific for reaction with sulfhydryl groups at neutral pH, another reacting partner could be an ε-amino group on a lysine residue having a low pK\textsubscript{a}. Such a group could also react with \( \alpha \)-keto acids through formation of a Schiff’s base. The possibility of Schiff’s base formation between pyruvate and a lysine residue on the alternative oxidase was examined using borohydride, which should reduce the Schiff’s base and irreversibly link pyruvate to the reactive lysine (e.g. Refs. 29 and 30). During alternative oxidase oxygen consumption by DTT-treated mitochondria in the presence of 5 mM pyruvate, NaBH\textsubscript{4} was added from a 0.5 M stock in 10 mM NaOH to a final concentration of 20 mM. After 5 min, the mitochondria were washed and assayed for pyruvate stimulation of alternative oxidase activity. Pyruvate stimulated the alternative oxidase activity of these mitochondria as much as washed control mitochondria that previously either had been exposed to borohydride alone, without pyruvate, or had been exposed to pyruvate but not borohydride (data not shown). The failure of borohydride to eliminate the reversibility of the stimulatory effect of pyruvate suggests that pyruvate does not activate the alternative oxidase through formation of a Schiff’s base.

DISCUSSION

The series of experiments presented in this paper indicate that sulfhydryl reagents can interact with the alternative oxidase protein at two different sites. One of the sites is the sulfhydryl involved in the redox-regulated intermolecular disulfide bond, as shown by the inability of diamide to oxidize the alternative oxidase of mitochondria isolated in the presence of iodoacetate or NEM (Fig. 2). The relative reactivities of the two sulfhydryl reagents toward this redox-active thiol are apparently different, however. NEM, was able to react with this group within the few minutes of an activity assay (Fig. 5), while iodoacetate required the longer time period of mitochondrial isolation to covalently bind at this site.

The second site of sulfhydryl reagent interaction with the alternative oxidase is at or near the site of pyruvate action. Both iodoacetate and NEM bind to this site during the short time period of an activity assay, block the interaction of pyruvate with the alternative oxidase, and have very different effects on alternative oxidase activity (Fig. 3). NEM is inhibitory, perhaps because of the large moiety that becomes bound to the enzyme. Similarly, the sulfhydryl reagent p-chloromercuribenzoic acid was reported to inhibit the *Sauromatum guttatum* alternative oxidase (31). Iodoacetate, on the other hand, was able to irreversibly stimulate the alternative oxidase when it reacted at this second site on the enzyme. The stimulation mimicked that of the reversible activation by pyruvate, because iodoacetate, like pyruvate, enhanced the alternative oxidase’s reactivity with reduced ubiquinone (Fig. 4). The replacement of pyruvate in this capacity by iodoacetate is most readily explained by iodoacetate’s site of action being the same as pyruvate’s, rather than simply in proximity. Thus, iodoacetate can affect the activity of the alternative oxidase in two ways: by maintaining the reduced state of the redox-active regulatory sulfhydryl/disulfide system and by reacting irreversibly at the pyruvate site of action.

The common site of pyruvate, iodoacetate, and NEM action is likely to be a sulfhydryl group for a number of reasons. Al
though NEM can react with amino groups (i.e., lysine) and iodoacetate can react with lysine, histidine, and methionine (32), reactivity of both reagents toward cysteine at neutral pH is nearly 1000-fold greater than with the other groups (32, 33). Both NEM and iodoacetate were able to rapidly modify the alternative oxidase at the pyruvate site under the neutral pH and short time period of an activity assay. Modification of non-sulfhydryl residues by these two reagents generally requires more alkaline pH and hours, rather than minutes, of exposure (32–34). While reaction at a lysine cannot be ruled out entirely, sulfhydryl residues are most likely the only ones to have been modified under the assay conditions used.

Additionally, there are two types of possible interactions of α-keto acids, such as pyruvate, with proteins. One is the formation of an imine between a lysine and the pyruvate α-carbonyl group. This Schiff’s base association can be stabilized by reduction with borohydride (29, 30). Because attempts to stabilize the pyruvate-alternative oxidase protein association with borohydride did not preserve the activated enzyme state or prevent subsequent pyruvate activation, the interaction of pyruvate with a lysine seems unlikely.

A second potential interaction between α-keto acids and proteins is the formation of a thiohemiacetal by reaction at a sulfhydryl residue (Refs. 35 and 36; Fig. 1C). Such an interaction has been suggested to be responsible for the inhibition of succinate dehydrogenase by oxaloacetate (37), which, like pyruvate, is an α-keto acid. Oxaloacetate associates with a sulfhydryl located within the active site of the enzyme, preventing the binding of succinate, the inhibitor malonate, or NEM (38, 39). Thus, a thiohemiacetal has a regulatory function in succinate dehydrogenase, albeit inhibitory in nature. Although not regulatory, a thiohemiacetal has also been shown to form following reaction of glyceraldehyde 3-phosphate with glyceraldehyde-3-phosphate dehydrogenase during the initial step in catalysis (40).

The formation of a thiohemiacetal is consistent with several properties of the pyruvate-alternative oxidase association. Pyruvate can be readily washed from mitochondria with a concomitant loss of stimulation (Ref. 20; this paper), consistent with the facile reversibility of a thiohemiacetal (35, 36), yet its interaction with the alternative oxidase is sufficiently stable to protect the site from NEM inhibition (Fig. 3). Another argument for the formation of a thiohemiacetal within the alternative oxidase is the similarity between the structure of the resulting carboxymethyl moiety added to sulfhydryls following reaction with iodoacetate and the thiohemiacetal formed by

![Figure 6](image)
The sulfhydryl responsible for redox regulation may be the more NH$_2$-terminal of the two conserved cysteines (Fig. 7). The binding of NEM to the redox-active sulfhydryls under conditions where the pyruvate site is first protected by iodoacetate caused essentially no change in activity (Fig. 6C). Failure of the bulky maleyl moiety to inhibit activity following reaction at the redox-active regulatory sulfhydryl site is consistent with this site being located distant from the catalytic center. Furthermore, thioredoxin may play a role in regulation of alternative oxidase activity (11, 45) and models of proteins subject to redox regulation through the action of thioredoxin predict that thioredoxin-modifiable residues will be distant from active sites and on the surface of the protein (in order to be accessible to thioredoxin (46). The more NH$_2$-terminal of the two conserved cysteines is in a very hydrophilic region of the protein (9), thus placing it in a potentially thioredoxin-accessible region.

The general effects of iodoacetate and pyruvate on the alternative oxidase from other source tissues are the same as those found with soybean cotyledon alternative oxidase. However, there are some differences in specifics. For the alternative oxidase of soybean root mitochondria, although pyruvate and iodoacetate both stimulate the enzyme, neither pyruvate nor iodoacetate protect the enzyme from inhibition by NEM (3). This may indicate that the thermogenic padix of its action on a different gene product from that (or those) expressed in cotyledons. Experiments with mitochondria isolated from aroid spadix (Symlocarpus foetidus and S. guttatus) indicate that the alternative oxidase from these sources also responds to pyruvate, iodoacetate, and NEM much like that of the soybean cotyledon oxidase. However, during the period of thermogenesis, the oxidase appears less responsive to pyruvate addition and, similarly, shows loss response to iodoacetate or NEM (3). This may indicate that the thermogenic spadix oxidase exists in a different native activation state from that of the soybean alternative oxidase or that these tissues express an isozyme that is “constitutively” activated and not as sensitive to pyruvate stimulation.

The two conserved cysteines make excellent targets for site-directed mutagenesis studies. Such studies could confirm the biochemically based results and interpretations presented here. In particular, mutation of the appropriate cysteine to an acidic residue such as glutamate could result in an alternative oxidase that behaves as if it were constitutively pyruvate-activated. Likewise, if the redox-active regulatory cysteine were also modified to an alanine, a permanently reduced, activated alternative oxidase might be obtained that could no longer be inactivated by formation of the disulfide-linked dimer.

Short of site-directed mutagenesis or protein purification, insight into the role of each cysteine might be gained by study of the alternative oxidase from non-plant sources. The alternative oxidase protein sequence from Pichia stipitis (formerly Hansenula anomala), a yeast, has a cysteine only a few residues displaced from the cysteine near the first membrane-spanning helix in the plant sequences (6), whereas no cysteine is found in this region in the Neurospora crassa sequence (7). Both the P. stipitis and N. crassa sequences have additional cysteines in the amino-terminal hydrophilic domain (6, 7), al-

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3 A. L. Umbach and J. N. Siedow, unpublished observations.
though analysis suggests these are not homologous with the more NH₂-terminal of the two conserved cysteines in the plant sequences. A comparison of the relative effects of the sulfhydryl reagents and pyruvate on the ability of each of these enzymes to be stimulated by pyruvate or to form an oxidized dimer might further suggest which cysteine is associated with pyruvate stimulation and which with covalent cross-linking.

Although the conclusions derived from the experiments presented in this study need to be furthered by the approaches described above, the importance of the two conserved cysteines in the plant alternative oxidase for its regulation and activity is clear. The two regulatory mechanisms are interrelated such that, although the redox-active, regulatory sulfhydryl may be reduced, the oxidase will not respond to low levels of reduced ubiquinone until an α-keto acid is present. Conversely, if pyruvate is present, significant alternative oxidase activation can only be achieved if the redox-active, regulatory cysteines are reduced. The interplay of these two forms of cysteine-based regulation produces a wide range of alternative oxidase activities governed by both metabolite levels and the redox poise of the mitochondrial matrix.

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