Plant Physiol. (1989) 89, 1311–1317
0032-0889/89, 89/1311/07/$01.00/0

Received for publication June 24, 1988
and in revised form September 1, 1988

Monoclonal Antibodies to the Alternative Oxidase of Higher Plant Mitochondria[1]

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ABSTRACT

The higher plant mitochondrial electron transport chain contains, in addition to the cytochrome chain which terminates with cytochrome oxidase, an alternative pathway that terminates with an alternative oxidase. The alternative oxidase of *Sauromatum guttatum* Schott has recently been identified as a cluster of proteins with apparent M_r of 37, 36, and 35 kilodaltons (kD). Monoclonal antibodies have now been prepared to these proteins and designated as AOA (binding all three proteins of the alternative oxidase cluster), AOU (binding the upper or 37 kD protein), and AOL (binding the lower or 36 and 35 kD proteins). All three antibodies bind to their respective alternative oxidase proteins whether the proteins are in their native or denatured states (as on protein blots). AOA and AOU inhibit alternative oxidase activity around 49%, whereas AOL inhibits activity only 14%. When coupled individually to Sepharose 4B, all three monoclonal resins were capable of retaining the entire cluster of alternative oxidase proteins, suggesting that these proteins are physically associated in some manner. The monoclonals were capable of binding similar mitochondrial proteins in a number of thermogenic and nonthermogenic species, indicating that they will be useful in characterizing and purifying the alternative oxidase of different systems. The ability of the monoclonal-Sepharose 4B resins to retain the cluster of previously identified alternative oxidase proteins, along with the inhibition of alternative oxidase activity by these monoclonals, supports the role of these proteins in constituting the alternative oxidase.

The plant mitochondrial electron transport chain consists in part of several substrate dehydrogenase complexes that reduce a common pool of the membrane lipid ubiquinone. This reduced pool of ubiquinone is then oxidized by either the classical cytochrome pathway that terminates with cytochrome oxidase or by the alternative pathway that terminates with the alternative oxidase. Both oxidases reduce oxygen to form water as a product (23). When electrons flow through the cytochrome pathway, energy is conserved in the form of an electrochemical gradient across the inner mitochondrial membrane. This gradient is then used to drive membrane-associated energy-dependent processes such as metabolite transport and ATP synthesis. In contrast, when electrons flow through the alternative pathway *per se*, no gradient is formed and the potential energy of the system is lost as heat. The quinone pool is preferentially oxidized by the cytochrome pathway, with electrons flowing into the alternative pathway to a greater extent when the cytochrome chain is either saturated, limited by the electrochemical gradient (respiratory control), or inhibited in some manner (12). Mitochondrial electron transport *in vivo* is usually limited by the electrochemical gradient, and as a result, when the alternative pathway is present it is often engaged to some extent depending upon the degree of respiratory control present. Engagement of the alternative pathway is also dependent upon the capacity of the substrate dehydrogenase complexes to feed electrons into the quinone pool. The capacity of these complexes depends upon the levels and activities of the complexes themselves, as well as the availability of substrate (4).

The alternative pathway is most highly expressed in thermogenic tissues, where its function is to generate heat used in various ways to attract insects, thereby facilitating pollination (10, 15–17). Expression of the alternative pathway in nonthermogenic tissues is often correlated with increased metabolic activity such as that observed in seedling development, fruit ripening, and in wound responses (1, 12, 19, 23). The alternative pathway in these tissues is likely expressed to allow for the continued generation of synthetic intermediates by the mitochondria when the cellular energy charge is high (9, 19, 23). Expression of the alternative pathway makes it possible to bypass normal respiratory control, allowing for the recycling of both mitochondrial matrix and cytoplasmic NADH. Expression of the alternative pathway is tissue specific, and within a tissue, under strict developmental control (2, 3, 10).

The alternative oxidase of *Sauromatum guttatum* Schott (voodoo lilies) has recently been sufficiently purified from mitochondria of the thermogenic appendix to allow for the identification of its polypeptide constituents as a cluster of proteins with apparent M_r of 37, 36, and 35 kD (3). The 37 kD protein appears constitutive in *Sauromatum*, whereas presence of the 36 and 35 kD proteins correlates with presence of alternative oxidase activity. These proteins were identified as components of the alternative oxidase, and other proteins may be involved in the alternative pathway or in regulatory roles. Polyclonal antibodies raised to the identified alternative oxidase proteins indicated that they were antigenically related. Expression of the 36 and 35 kD proteins was found to be under the control of calorigen (3). Calorigen is produced in the anthers of *Sauromatum* and migrates into the appendix region where it triggers thermogenesis (15). An active com-

[1] Supported in part by National Science Foundation postdoctoral fellowship (T. E. E.) grant DMB-8508782 and Department of Energy contract DE-AC02-76ERO-1338.

ponent of extracts of *Sauromatum* anthers was recently established to be salicylic acid by Raskin *et al.* (20).

We have now produced monoclonal antibodies to the 37, 36, and 35 kD proteins, and in this paper present results obtained with the monoclonals which further support the view that these proteins constitute the alternative oxidase. The monoclonal antibodies obtained are widely cross-reactive, and thus will be useful in investigating the alternative oxidase of organisms other than *Sauromatum guttatum*.

MATERIALS AND METHODS

Plant Material

Sauromatum guttatum Schott (voodoo lilies) were maintained as previously described (3). Mitochondria isolated from various regions of the thermogenic inflorescence were used in this study. Each inflorescence consists of a spadix surrounded by a spathe. The day that the appendix region of the spadix heats is referred to as 'D-day' (15), and other developmental stages of the inflorescence are indicated as the number of days before or after D-day. Eastern skunk cabbage (*Symplocarpus foetidus* L.) spadices were collected in Michigan. *Amorphophallus rivieri* Dur. and *Arum italicum* Mill. spadices were generous gifts of Bastiaan Meeuse (Botany Department, University of Washington, Seattle). Mung bean (*Vigna radiata* L.) seedlings were grown in darkness in moist vermiculite at 30 ± 2°C for 5 d. Potato tubers (*Solanum tuberosum* L. cv Russet) and avocado fruits (*Persea americana* Mill.) were obtained at local markets. Tobacco callus (*Nicotiana tabacum* vs Petite Havana SR1) was grown in the dark at 28°C on Murashige-Skoog salt base (KC Biological) supplemented with Gamborgs B-5 vitamins, 3.0% (w/v) sucrose, 0.8% (w/v) phytagar, 1×10^{-5} M napthalene acetic acid, and 1×10^{-6} M 6-benzyladenine (7, 13, 14, 18).

Mitochondrial Isolation and Preparation of Solubilized Alternative Oxidase Fractions

Washed mitochondria were isolated from all tissues with the procedure of Schwitzguebel and Siegenthaler (22). The isolated mitochondria were resuspended in 250 mM sucrose/30 mM Mops[2] (pH 6.8). BIGCHAP-solubilized alternative oxidase was prepared from these mitochondria as before (2) and subsequently used for preparation of CM-Sepharose and phenyl-Sepharose alternative oxidase fractions (3). CM-Sepharose fractions were often combined and concentrated above an Amicon YM-100 membrane before use.

Assays

Oxygen uptake assays were performed at 25°C in 1.0 mL of medium using a Rank Brothers electrode. The oxygen content of air-saturated water was estimated according to Estabrook (5). Capacities of the cytochrome and alternative pathways were determined as before (4). The alternative oxidase was assayed in BIGCHAP buffer (0.5% BIGCHAP/1 mM EDTA/30 mM Mops, pH 6.8). The quinone analog durohydroquinone was used as the artificial substrate. Alternative oxidase activity was measured as that durohydroquinone-dependent oxygen uptake that was sensitive to an inhibitor of the alternative pathway, SHAM. Duroquinone was reduced to durohydroquinone by the method of Rich (21). Protein was measured by means of a modified Lowry (11). Gel electrophoresis, protein blots, and antibody probing of protein blots were performed as previously described (3).

Antibody Production, Purification, Conjugation to Alkaline Phosphatase, and Subtyping

Polyclonal antibodies were raised in BALB/c female mice (6). Monoclonal antibodies were prepared from these mice essentially as described by Galfre and Milstein (6). Myeloma line SP2/O-Ag14 (6) was used for fusion with the spleen cells. Culture media from resulting hybridomas were initially screened for useful monoclonals with protein blots. This evaluated the potential of any antibodies being produced to successfully bind the denatured form of the alternative oxidase proteins. Hybridomas producing monoclonals that were positive on protein blots were then screened with ELISAS. The ELISAS were designed to evaluate the ability of the antibodies to bind the native form of the protein. Hybridomas that produced antibodies recognizing both the native and denatured forms of the proteins were cloned twice by limiting dilution. Once established, the hybridoma lines were grown in a medium consisting of Ventrex (Portland ME) plus 2% (v/v) fetal calf serum, 2 mM L-glutamine, 25 mg ampicillin, and 100 mg streptomicin sulfate per 1, and 0.1% (v/v) Fungizone (GIBCO). All lines have been grown continuously in this medium for over a year while maintaining good vigor. Monoclonal antibodies were purified from hybridoma supernatants using ammonium sulfate precipitation (0–50% [v/v] precipitate) and hydroxylapatite chromatography (24). Alkaline phosphatase conjugates of all monoclonals were prepared according to Voller *et al.* (25). Each monoclonal was subtyped with Ouchterlony immunodiffusion plates (Serotec, Blackthorn, UK) and with an ELISA-based kit (HyClone, Logan, UT).

ELISAS

ELISAS were designed to test for the ability of monoclonals to bind to alternative oxidase proteins in their native form. Nunc-Immuno Plate I 96-well plates were used, and all steps were performed at room temperature. Concentrated CM-Sepharose fractions were used as the antigen source for coating the wells. The CM-fractions contain a number of proteins, including those of the alternative oxidase (3). Approximately 5 μg of CM-fraction protein was added per well in 50 μL of Mops buffer (10 mM Mops, 150 mM NaCl, pH 7.2), followed by a 1 h incubation to allow for binding of the antigens to the wells. The antigen solution was then removed, and the wells blocked through a 1 h incubation with 50 μL of Mops buffer

[2] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; BIGCHAP, *N,N-bis*-(3-D-glucoamidopropyl)deoxycholamide; CM, carboxymethyl; SHAM, salicylhydroxamic acid; ELISA, enzyme-linked immunosorbent assay; AOA, alternative oxidase monoclonal that binds the 37, 36, and 35 kD proteins; AOU, alternative oxidase monoclonal that binds the 37 kD protein; AOL, alternative oxidase monoclonal that binds the 36 and 35 kD proteins; natoms 0, (2) (nmol O_2).

containing 1 mg/mL BSA. The blocking solution was removed and hybridoma culture media to be tested (50 μL) were added for a 1 h incubation. The culture supernatants were removed, and antimouse-alkaline phosphatase (50 μL of a 1/1000 dilution in Mops buffer) was added for 1 h, followed by three washes with 100 μL of Mops buffer. Color was developed in 50 μL of 5 mM *p*-nitrophenyl phosphate in 100 mM Trizma Base/100 mM NaCl/and 5 mM $MgCl_2$ (pH 9.5). After a suitable degree of color development, the wells were read at 405 nm. Wells were read three times, with positive responses taken as absorbance changes between time intervals that were significantly over background absorbance changes.

Preparation and Use of Monoclonal Resins

Monoclonal antibodies were coupled to cyanogen bromide activated Sepharose 4B according to Goding (8). Approximately 1 mg of purified antibody was coupled to 100 μL of resin. Antibody resins were stored and used at 4°C. They were precycled prior to each use with a 30 min wash in elution buffer (5 mM glycine-HCl, pH 2.3/100 mM NaCl/0.5% BIGCHAP), followed by two washes in BIGCHAP buffer plus 100 mM NaCl. Resins were incubated with concentrated CM-Sepharose fractions for 12 h. The resins were separated by centrifugation and washed twice with BIGCHAP buffer plus NaCl. Proteins bound to the antibody resins were then eluted by incubation with elution buffer for 30 min. After use, the resins were washed twice with PBS before storage.

RESULTS AND DISCUSSION

Production of Monoclonal Antibodies to the Alternative Oxidase

In a recent paper (3), the alternative oxidase of *S. guttatum* was shown to consist of a cluster of proteins with apparent M_r of 37, 36, and 35 kD. Polyclonal antibodies raised in mice were used to establish that these proteins are antigenically related (3). We have now sacrificed these mice for the preparation of monoclonal antibodies in an effort to obtain potentially useful monoclonals for further investigations of the alternative oxidase. Spleen cells from the mice were fused with tumor cells (SP2/0-Ag14) resulting in the formation of hybridomas. The hybridomas secrete monoclonal antibodies into the culture media, and a few of these will be producing antibodies to alternative oxidase proteins. Culture media from hybridomas were initially screened for useful monoclonals using protein blots of total mitochondrial proteins (Fig. 1). This evaluated the potential of any monoclonals present to bind the denatured or linear form of the proteins. The first lane of Figure 1 shows the results of probing with polyclonal antibodies raised to the 36 kD protein (3). Anti-36 kD protein polyclonal antibodies bind the 37, 36, and 35 kD alternative oxidase proteins. Three different classes of monoclonals were obtained to the alternative oxidase proteins. These are represented in the monoclonal lanes of Figure 1. Monoclonals which bound to all three of the identified alternative oxidase proteins were called AOA (Alternative Oxidase All). Those that bound only the upper or 37 kD protein were designated as AOU, and those binding to the lower proteins (36 and 35 kD) as AOL.

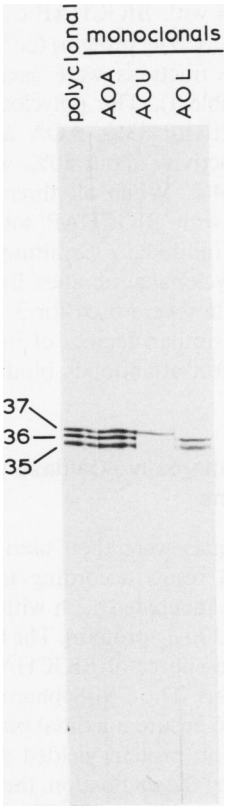

Figure 1. Protein blots probed with antibodies to the alternative oxidase. Protein blots are of total mitochondrial proteins from D-1 appendix tissue (approximately 20 μg per lane). Polyclonal serum was used at a 1/1000 dilution and monoclonal antibodies as 1/20 dilutions of hybridoma culture supernatants in PBS. Results are representative of numerous antibody probings. Apparent M_r in kD are indicated on the left.

Hybridomas producing desirable monoclonals based on protein blots were then screened with ELISAS which were designed to evaluate the potential of the monoclonals to bind the native form of the proteins. Monoclonals that recognize both the linear and native form of proteins are the most desirable since they will be useful for essentially all types of antibody assays. The most ELISA positive hybridoma of each of the three classes was selected as these hybridomas were cloned twice by limiting dilution. During the cloning process, care was taken to screen a number of the most vigorous colonies for antibody production, selecting the best colonies at each cloning step. The monoclonals represented in Figure 1 are the three lines that resulted from this cloning and screening process. All three recognize both the native and linear forms of the proteins. All three were also determined to be of the subclass IgG_1.

Effect of the Monoclonal Antibodies on Alternative Oxidase Activity

Antibodies were partially purified through ammonium sulfate precipitation (0–50% cut) and hydroxylapatite chromatography. This allowed for quantitation of their effects on alternative oxidase activity. Purified antibodies (100 μg pro-

tein) were incubated with BIGCHAP solubilized alternative oxidase preparations (330 μg protein) for 5 h at 4°C. Following this incubation, the fractions were assayed for alternative oxidase activity (Table I). The polyclonal antibodies were found to inhibit activity 83%. AOA and AOU inhibited alternative oxidase activity about 50%, whereas AOL inhibited activity only 14%. When all three monoclonals were incubated together with BIGCHAP solubilized alternative oxidase, activity was inhibited 73%, almost to the same extent as observed with polyclonal antibodies. Experiments in which 50 μg of the antibodies were used for 3 h incubations (data not shown) yielded similar degrees of inhibition, indicating that the maximal effect of antibody binding was observed in the data of Table I.

Purification of the Alternative Oxidase with Monoclonal-Sepharose 4B Resins

Purified monoclonals were then used to prepare monoclonal-Sepharose 4B resins according to Goding (8). The resins (100 μL) were incubated 12 h with concentrated CM-Sepharose fractions (200 μg protein). The CM-Sepharose fractions are an enriched source of BIGCHAP solubilized alternative oxidase proteins. The CM-Sepharose fraction proteins were used in excess to ensure maximal binding, 100 μg of the CM-Sepharose fraction protein yielded similar results (data not shown). Following the incubation, the resins were washed and the proteins eluted with low pH. The eluted proteins were ammonium sulfate precipitated (0–60% cut) and subsequently used to run SDS-PAGE (Fig. 2). Included in Figure 2 are protein gels of the purification scheme previously published (3). The phenyl-Sepharose lane shows enrichment of the 37, 36, and 35 kD proteins. Antibody resins lanes represent protein gels of the proteins eluted from the monoclonal resins. All three monoclonal resins retained the 37, 36, and 35 kD proteins even though AOU binds only to the 37 kD protein and AOL binds only to the 36 and 35 kD proteins. AOA retained the proteins to an equivalent degree, whereas AOU retained the 37 kD protein more strongly, and AOL preferentially retained the 36 and 35 kD proteins. These results suggest that the alternative oxidase proteins are physically associated in some manner. Some antibody was often released from the resin by the low pH treatments. The bands

Table I. *Inhibition of Alternative Oxidase Activity with Antibodies*

Purified antibodies (100 μg) were incubated with 200 μL of BIGCHAP-solubilized alternative oxidase (average of 330 μg protein) for 5 h at 4°C. Following the incubation, these samples were assayed for alternative oxidase activity. A similar incubation containing an appropriate volume of PBS and 200 μL of BIGCHAP-solubilized alternative oxidase was used to establish the control rate of activity which averaged 60.1 natoms O/min/mg protein. The results are the mean of three experiments.

Antibody	Inhibition
	%
Polyclonal	83
AOA	50
AOU	48
AOL	14
AOA + AOU + AOL	73

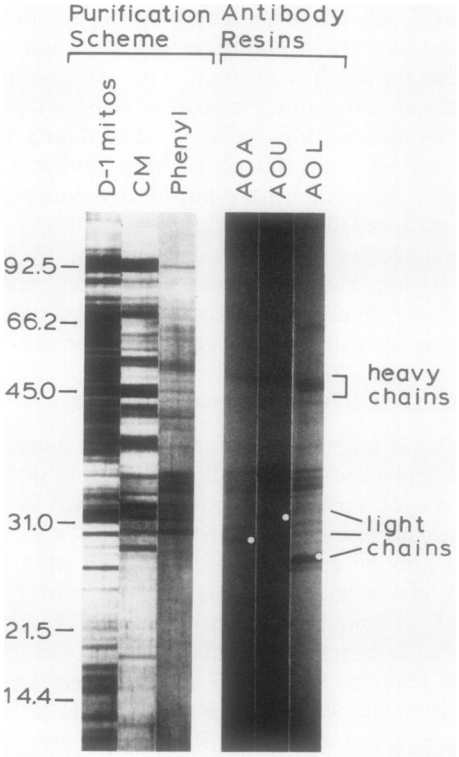

Figure 2. Purification of the alternative oxidase with monoclonal antibody resins. The purification scheme lanes represent protein gels of the alternative oxidase purification previously described, which is based upon a combination of cation-exchange (CM-Sepharose) and hydrophobic-interaction (phenyl-Sepharose) chromatography (3). The phenyl-Sepharose lane represents the degree of purification that was achieved with these columns. Monoclonal antibody resins that have now been prepared, yielded a similar degree of purification as shown by the protein gels represented in the antibody resins lanes. Protein bands corresponding to chains of the antibodies are indicated by arrows at the right of the figure. Apparent M_r in kD are indicated on the left. The results are representative of three experiments.

in these gels corresponding to the chains of the antibodies have been indicated by arrows and white dots. A few other protein bands are also sometimes present. These may represent other proteins associated with the alternative pathway, degraded alternative oxidase proteins, or proteins nonspecifically bound to the resins. Through comparisons of the protein profiles of CM-Sepharose fractions with the profiles of eluted proteins from several antibody resin preparations, it is apparent that the only proteins consistently enriched by the resins are the 37, 36, and 35 kD proteins. The enrichment of these proteins on monoclonal resins, along with inhibition of alternative oxidase activity by the monoclonals, support previous results indicating that the 37, 36, and 35 kD proteins constitute the alternative oxidase of *Sauromatum*. Whether or not other proteins are associated with the alternative pathway has not been established. These could potentially be involved in linking the identified alternative oxidase proteins to the electron transport chain, or they could be regulatory in nature.

Alkaline-Phosphatase Conjugates and 'Quick' Antibody Probings with Monoclonals AOA, AOU, and AOL

Alkaline-phosphatase conjugates were prepared with the purified monoclonals according to Voller *et al.* (25). Conju-

gation of alkaline-phosphatase to AOA did not significantly impair the affinity of the antibody based upon antibody probing of protein blots. Thus, AOA-alkaline phosphatase conjugates can be use to save time during antibody probings. In contrast, alkaline-phosphatase conjugates of AOU and AOL bound poorly to protein blots, indicating that the affinity of these antibodies was decreased considerably upon conjugation (data not shown).

Antibody probings can also be performed quickly if the primary and secondary antibodies are incubated at the same time together with the antigen. When AOA is added along with anti-mouse alkaline-phosphatase, the antibodies successfully bind to the alternative oxidase proteins. Thus, antibody bindings can be accomplished in as short a time as 10 min. If either AOU and AOL are used together with anti-mouse alkaline-phosphatase, the antibodies do not bind to the alternative oxidase proteins (data not shown). Binding of the secondary antibody to AOU and AOL diminishes their affinity for the alternative oxidase proteins to a similar degree as does the conjugation of these monoclonals to alkaline-phosphatase.

Cross-Reactivity of the Monoclonals to Mitochondrial Proteins of Other Thermogenic Systems

The monoclonals obtained to the alternative oxidase proteins of *Sauromatum* would be of greater value if they cross-reacted to the alternative oxidase proteins of other systems. We initially characterized the monoclonals using mitochondria from different thermogenic inflorescences (Fig. 3). In *Sauromatum*, *Arum*, and *Amorphophallus*, mitochondria from the appendix regions have high levels of alternative oxidase activity, while in *Symplocarpus* the petals have high activity. The protein blots probed with polyclonal antibodies are similar to those previously published (3), and show that the polyclonals cross-react with similar proteins in the thermogenic systems evaluated. AOA was found to essentially duplicate the binding characteristics of the polyclonal antibodies on protein blots. What appears to be a number of degradation products of these proteins appear in a number of the lanes. AOU also binds well to representative proteins in the different species, whereas AOL binds strongly only to proteins of *Sauromatum* and *Arum* (data not shown).

Cross-Reactivity of Monoclonal Antibody AOA to Mitochondria Proteins of Nonthermogenic Species

The ability of AOA to mimic the polyclonal antibodies on protein blots indicates that it may find general use for the identification of the alternative oxidase proteins. To evaluate this further, we have used AOA to probe total mitochondrial protein blots from four nonthermogenic species (Fig. 4). A lane of *Sauromatum* appendix mitochondria was included in Figure 4 as a reference. The results clearly indicate that AOA is capable of cross-reacting with similar proteins in the nonthermogenic species evaluated, and thus AOA may be useful for investigating the alternative oxidase in many species. What appears to be degradation products of the alternative oxidase proteins are prevalent in the potato and tobacco lanes. Whether this occurs normally *in vivo*, or whether it occurs during isolation of the mitochondria has not been addressed.

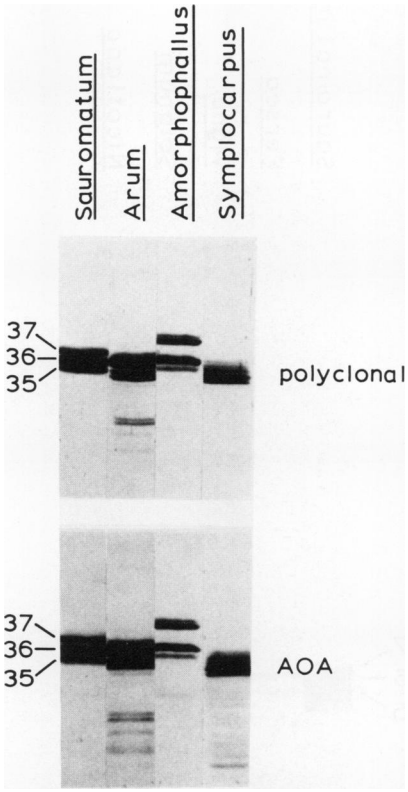

Figure 3. Usefulness of the alternative oxidase monoclonal antibodies in investigating other thermogenic systems. This figure represents a series of total mitochondrial protein blots probed with either polyclonal or monoclonal AOA antibodies to the alternative oxidase proteins. In *Sauromatum*, *Arum*, and *Amorphophallus*, mitochondria were isolated from the appendix regions of the inflorescences. In *Symplocarpus*, mitochondria were isolated from the petals. An average of 40 μg of mitochondrial protein was loaded per lane. Polyclonal antibodies were used as a 1/1000 dilution of serum and monoclonal antibodies as a 1/100 dilution of hybridoma supernatant in PBS. Apparent M_r in kD are indicated on the left. Results are representative of two experiments.

CONCLUSIONS

Considerable evidence now exists which indicates that the 37, 36, and 35 kD proteins of *Sauromatum* constitute the alternative oxidase. Purification of alternative oxidase activity results in a fraction enriched in these three proteins (along with a fourth protein with an apparent M_r of 30 kD). Presence of these proteins in mitochondria correlates with alternative oxidase activity in both a tissue specific and developmental fashion. Induction of alternative oxidase activity in *Sauromatum* with calorigen results in greatly increased expression of the 36 and 35 kD proteins. And finally, monoclonal antibodies that bind specifically to these proteins inhibit alternative oxidase activity.

The 3 alternative oxidase proteins were shown to be antigenically related through the use of polyclonal antibodies raised to each protein individually (3). Further support for their relatedness comes from the ability of a single monoclonal antibody (AOA) to bind all of these proteins. The three alternative oxidase proteins may be physically associated because antibody resins, prepared from monoclonals that differ in their ability to bind the three proteins, retain the entire

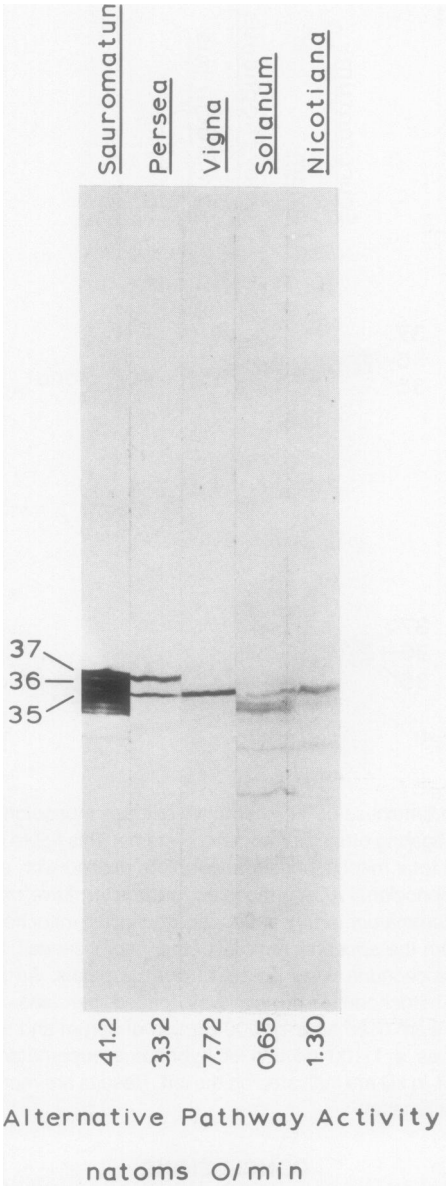

Figure 4. Cross-reactivity of monoclonal antibody AOA to mitochondrial proteins of nonthermogenic species commonly used to investigate the alternative oxidase. The lanes represent total mitochondrial protein blots probed with monoclonal AOA (1/100 dilution in PBS). The alternative pathway activity presented at the bottom of each lane corresponds to the amount of total mitochondrial protein present, which was for *Sauromatum* D-day appendix (50 μg), *Persea* unripe fruit (100 μg), *Vigna* etiolated hypocotyls (200 μg), *Solanum* fresh tuber (300 μg), and *Nicotiana* callus (200 μg). Results are representative of two experiments; M_r in kD are indicated on the left.

cluster. Two of these monoclonals (AOA and AOU) bind to these proteins in a manner that results in significant inhibition of alternative oxidase activity. When AOL binds, it apparently does not affect the active site(s) of the alternative oxidase proteins. AOA will likely find the widest general use for identifying alternative oxidase proteins. It recognizes both the native and denatured forms of the proteins, its affinity is not greatly affected by conjugation to alkaline-phosphatase or by binding to a secondary antibody, and it is widely cross-reactive.

ACKNOWLEDGMENTS

Thanks to Bastiaan Meeuse for his advice and for providing us with most of the plant material used. We thank Shauna C. Somerville, Cindy Garbus-Gooch, and Allen D. Smith for their help with the production of monoclonal antibodies. We also thank Kristin A. Rorrer for providing us with tobacco callus.

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