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# Alternative Oxidase of Potato Is an Integral Membrane Protein Synthesized *de Novo* during Aging of Tuber Slices[1]

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## ABSTRACT

**The rise in alternative respiratory capacity upon aging of potato (*Solanum tuberosum*) tuber slices is correlated with changes in mitochondrial membrane protein composition and a requirement for cytoplasmic protein synthesis. However, the lack of an antibody specific to the alternative oxidase has, until recently, prevented examination of the alternative oxidase protein(s) itself. We have employed a monoclonal antibody raised against the *Sauromatum guttatum* alternative oxidase to investigate developmental changes in the alternative pathway of aging potato slice mitochondria and to characterize the potato alternative oxidase by one- and two-dimensional gel electrophoresis. The relative levels of a 36 kilodalton protein parallel the rise in alternative path capacity. A plausible interpretation is that this alternative oxidase protein is synthesized *de novo* during aging of potato slices.**

In addition to the cyanide-sensitive Cyt pathway of electron transport, plant mitochondria possess a cyanide-resistant, hydroxamate-sensitive alternative pathway. Both pathways transfer electrons from ubiquinone to oxidases that reduce oxygen to $H_2O$ (31). In the thermogenic inflorescences of species of the Araceae, electrons are channeled through the alternative pathway to generate heat, which volatilizes compounds to attract insect pollinators (2, 9, 21, 22). The purpose of the alternative pathway in nonthermogenic species is unknown. One view is that electrons flow through the alternative pathway whenever the Cyt pathway is saturated or limited (the overflow hypothesis), permitting continued functioning of the TCA cycle to process carbon skeletons even when ATP is not needed and using up excess carbohydrates (reviewed in 15, 20, 26). In *Neurospora*, the alternative oxidase proteins are not constitutively synthesized, but when the synthesis of mitochondrially encoded components of the Cyt pathway is inhibited by chloramphenicol, the synthesis of the nuclear-encoded alternative oxidase proteins is induced (16). Studies on the mechanism of electron partitioning between the two pathways in nonaroid higher plants have been hindered by the lack of specific antibodies or genetic probes for the alternative oxidase. However, with the isolation of antibodies to the alternative oxidase (8), a classic system for alternative pathway induction—aging potato tuber slices—presents itself as a model system for such future studies.

The wound respiration of potato (*Solanum tuberosum*) was described as early as 1887 (referenced in 11), and the cyanide-resistance of aged slice respiration was recognized and investigated in the late 1940s and early 1950s (11). Initially, it was suggested that such respiration may be the result of free radical reactions (reviewed in 31) or due to the activity of lipoxygenase (23). However, that the alternative oxidase is a lipoxygenase (which also consumes oxygen and is cyanide-insensitive) associated with membrane lipid breakdown or repair has been ruled out by careful isolation of mitochondria (25). The possibility that cyanide-resistant respiration may be nonenzymatic in nature has also been ruled out through immunological data demonstrating the presence of specific proteins necessary for activity and by analysis of the engagement of the alternative pathway in whole tissues (7–10). Fresh tissue slices from tubers demonstrate respiration that is sensitive to cyanide while aged potato tuber slices exhibit cyanide-resistant respiration (3, 11, 29). However, the induction of alternative pathway respiration in aged slices could be mimicked in fresh slices if the tubers were treated with ethylene, ethanol, acetaldehyde, acetate, or cyanide for 24 h before slicing (1, 13, 14, 24, 27). These results have been interpreted to mean that the alternative pathway is present constitutively in tubers (20). Theologis and Laties (28, 30) proposed that membrane integrity is necessary for the functioning of the alternative oxidase and that slicing causes mitochondrial membrane breakdown which is repaired during aging.

Changes in mitochondrial membrane protein composition (4) and a requirement for cytoplasmic protein synthesis (3) have been correlated with the rise in alternative respiratory capacity in aging potato slices. Until recently, the lack of an antibody specific to the alternative oxidase has prevented examination of the levels of the alternative oxidase protein(s) itself. We show here that a monoclonal antibody raised against the *Sauromatum guttatum* alternative oxidase (8) can be employed to characterize developmental changes in the potato alternative pathway. The alternative oxidase was partially purified and characterized by two-dimensional gel electrophoresis. It is interesting to note that the relative levels of the protein parallel the rise in alternative oxidase capacity in potato. Similar results have been previously published for *S. guttatum* (7–9) and the fungal alternative oxidase from *Neurospora crassa* (16).

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## MATERIALS AND METHODS

### Aging of Potato Slices and Isolation of Mitochondria

Potatoes (*Solanum tuberosum* cv Russet Burbank) were obtained from Dr. David Douches (Department of Crop and Soil Sciences, Michigan State University). Aging of potato slices for 12 or 24 h was carried out according to Dizengremel and Lance (3) in the presence of 50$\mu$g/mL chloramphenicol. Aged slices or shredded fresh potatoes were blended in 1.5 volumes of isolation buffer (400 mM sorbitol, 30 mM MOPS, 1 mM EDTA, 4 mM cysteine, 0.2% BSA, 0.6% polyvinyl polypyrrolidone [pH 7.6]). After filtration through four layers of sterile Miracloth (Calbiochem), the homogenate was centrifuged at 1,400$g$ for 5 min. The resulting supernatant was centrifuged at 16,300$g$ for 15 min to pellet the mitochondria. The pellets were resuspended in wash buffer (350 mM sucrose, 30 mM MOPS, 1 mM EDTA, 0.2% BSA [pH 7.2]), and the suspension was centrifuged at 4,300$g$ for 2 min. The supernatant was centrifuged at 20,000$g$ for 5 min to pellet the mitochondria. The mitochondria were further purified by sucrose density gradient centrifugation (5).

### Respiration Assays

Purified mitochondria were resuspended in assay buffer (500 mM mannitol, 10 mM $K_2HPO_4$, 10 mM KCl, 5 mM $MgCl_2$ [pH 7.2]). Oxygen uptake was measured in a Rank Brothers electrode apparatus (digital oxygen system model 10) at 25°C in a total volume of 1.0 mL. Samples contained 0.1 to 0.3 mg protein, as determined by a modified Lowry method (19). The exogenous substrate used was 10 mM succinate with 150 $\mu$M ADP, then mitochondria were uncoupled with 0.5 $\mu$M FCCP[2] before respiration measurements were taken. Cyt pathway capacity was taken to be that portion of the respiration inhibited by 1 mM KCN. Alternative pathway capacity was measured as that portion of the respiration inhibited by 1 mM SHAM in the presence of 1 mM KCN (9). Residual respiration is the oxygen uptake which remained in the presence of 1 mM KCN + 1 mM SHAM (17).

### Partial Purification of Membrane Proteins and Immunoblotting

The alternative oxidase and other mitochondrial membrane proteins were solublized and partially purified as described (6). Samples of mitochondria (400 $\mu$g protein) or partially purified membrane proteins (600 $\mu$g protein) were electrophoresed and transferred to nitrocellulose as previously described (7). The nitrocellulose filter was washed for 30 min in PBS-Tween (10 mM $NaH_2PO_4$, 150 mM NaCl [pH 7.2] with 0.3% Tween-20), then reacted with a 1:200 dilution of the monoclonal antibody against the *Sauromatum guttatum* alternative oxidase (AOA; 8) in PBS-Tween for 1.5 h. After two 5-min washes with PBS-Tween, the filter was reacted for 1 h with a 1:1000 dilution of anti-mouse IgG-alkaline phosphatase conjugate in PBS-Tween (8). The filter was then washed for 5 min with PBS-Tween, then for 5 min with pH 9.5 buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ [pH 9.5] with 0.3% Tween-20). Color was developed by reaction with 3.3 mg nitroblue tetrazolium and 1.7 mg 5-bromo-4-chloro-3-indoyl phosphate per 10 mL of pH 9.5 buffer until the desired darkness was reached. The filter was then washed for 5 min in PBS-Tween followed by 10 min in PBS-Tween containing 5 mM EDTA.

### Two-Dimensional Gel Electrophoresis and Blotting

Isoelectric focusing of partially purified and solubilized aged potato slice mitochondrial membrane proteins (1.47 mg protein in 20 $\mu$L) was performed as described (12). Electrophoresis in the second dimension through 1.5 mm thick SDS-polyacrylamide gradient gels and immunoblotting were performed essentially as described above. Isoelectric focusing and two-dimensional gel electrophoresis of *S. guttatum* mitochondrial membrane proteins (60 $\mu$g in 20 $\mu$L) were performed as previously described (12). Blots were stained for total protein using 100 $\mu$L India ink in 20 mLs PBS-Tween.

## RESULTS

Figure 1 presents the results of respiration assays on mitochondria isolated from fresh, 12-h-aged, and 24-h-aged potato tissue. The rate of uncoupled respiration (averages of three experiments) rose from 109 ± 46 natom O $min^{-1}$ (mg protein)$^{-1}$ in fresh potato slice mitochondria to 181 ± 32 natoms O $min^{-1}$ (mg protein)$^{-1}$ after 12 h of aging. The rate then declined after 24 h of aging to 89 ± 15 natoms O $min^{-1}$ (mg protein)$^{-1}$. The percentage of the total respiratory capacity, that was cyanide-sensitive and mediated by the Cyt pathway decreased from 84.1 to 42.7% with aging (Fig. 1). The percentage that was cyanide-resistant, SHAM-sensitive, and mediated by the alternative pathway increased from 6.7 to 38.1%. Residual respiration, insensitive to both cyanide and SHAM and not mediated by either pathway, also increased with aging. These results agree with other reports of cyanide-insensitive respiration in potato slices (11, 29) and isolated mitochondria (3, 25).

The differences in alternative oxidase capacity presented in Figure 1 are reflected in the levels of alternative oxidase protein. A monoclonal antibody raised against the *S. guttatum* alternative oxidase (8) was used on Western blots of mitochondrial proteins to determine the presence and relative concentration of the alternative oxidase. Mitochondria were isolated from fresh and 24-h-aged potato tissue and membrane proteins partially purified as described in "Materials and Methods." Whole mitochondria, supernatant fractions (containing BIGCHAP-solublized membrane proteins), and pellet fractions (containing membrane proteins insoluble in BIGCHAP) are compared in Figure 2. The alternative oxidase antibody reacted to several low mol wt (12–18 kD) polypeptides in fresh potato mitochondria (lane A2), but to one polypeptide of an apparent mol wt of 36 kD (similar to the *S. guttatum* alternative oxidase) in 24-h-aged potato mitochondria (lane B2). The low mol wt polypeptides were faintly present in aged mitochondria while the high mol wt polypep-

[2] Abbreviations: FCCP, *p*-trifluoromethoxycarbonylcyanide; SHAM, salicylhydroxamic acid; Tween 20, polyoxyethylenesorbitan monolaurate; BIGCHAP, *N,N*-bis-(3-D-glucoamidopropyl)-deoxycholamide.

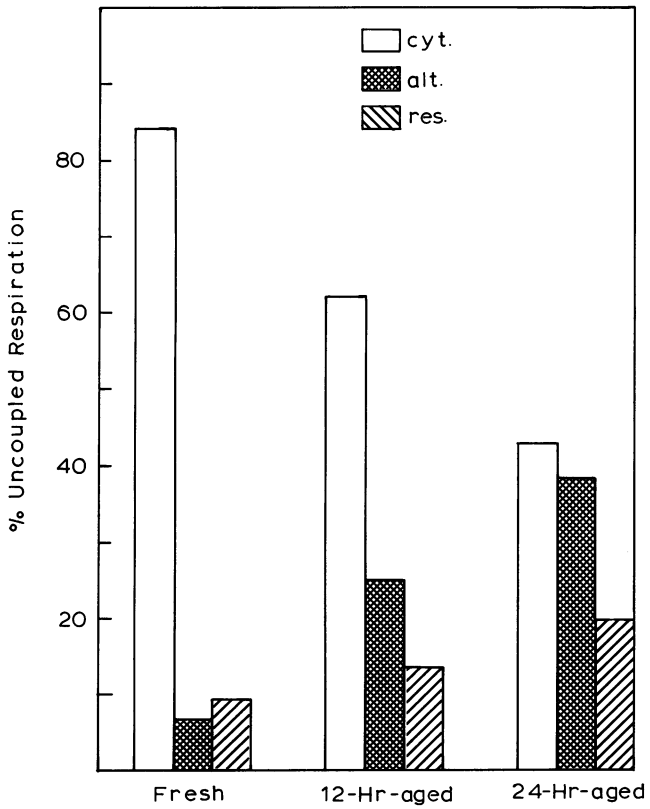


**Figure 1.** Respiration of potato mitochondria. Oxygen consumption of mitochondria isolated from fresh, 12-h-aged, and 24-h-aged potato discs was measured as described in "Materials and Methods." White bars represent Cyt pathway capacity, shaded bars represent alternative pathway capacity, and striped bars represent residual respiration. Capacities are expressed as the percentage of the uncoupled, uninhibited respiration rate and are the averages of three experiments with at least three runs per experiment.

tide was absent or present in extremely low concentration in fresh mitochondria. The low mol wt polypeptides also appeared in the pellet fractions of both fresh (lane A3) and 24-h-aged (lane B3) membrane protein preparations but were not present in the supernatant fractions (lanes A4 and B4). These polypeptides were apparently not easily solubilized or removed from the mitochondrial membranes. The high mol wt polypeptide was not present in the supernatant fraction of the fresh membrane protein preparation (lane A4), but was enriched in the 24-h-aged membrane protein preparation (lane B4). We believe this high mol wt polypeptide is the alternative oxidase of potato or one component of it, and the lower mol wt polypeptides may be degradation products.

These data suggest that the increased alternative pathway capacity in aged tissue results from an increased level of alternative oxidase protein. It has been previously shown in *S. guttatum* that increased alternative pathway capacity is associated with increased levels of the alternative oxidase proteins (7–9). Cytoplasmic protein synthesis is required for the development of alternative path capacity in aging potato slices (3). Therefore, the increased levels of alternative oxidase protein in aged sliced mitochondria may be due to *de novo* synthesis during aging of the 36 kD active alternative oxidase which was not present in mitochondria of whole tubers or nonaged slices. Of course, our experiments do not differentiate between increased synthesis or decreased turnover.

To test the remote possibility that the lower mol wt bands were degradation products caused by exposure of previously sequestered protein or by an SDS-activated protease during preparation of samples for gels, an experiment was performed in which *S. guttatum* appendix mitochondria containing high amounts of alternative pathway were mixed with potato mitochondria (Fig. 3). Some samples were boiled immediately after the addition of the mitochondria to the sample buffer (left half of figure), while replicate samples were incubated at room temperature for 10 min in the sample buffer before boiling (right half of figure). If there were a protease active during sample preparation, then in the incubated samples it might have been expected to degrade the three prominent *S. guttatum* bands normally seen on Western blots probed with the alternative oxidase antibody. However, identical bands were visible in both the incubated and the nonincubated samples. No bands were seen in the *S. guttatum* plus potato samples (lanes 3 and 8) that were not also present in samples of *S. guttatum* (lanes 6 and 11) or potato (lanes 2, 4, 7, and 9) mitochondria alone. This experiment suggests that the low mol wt bands prominent in fresh potato mitochondria are not a result of general degradatory processes or an SDS-activated protease during sample treatment.

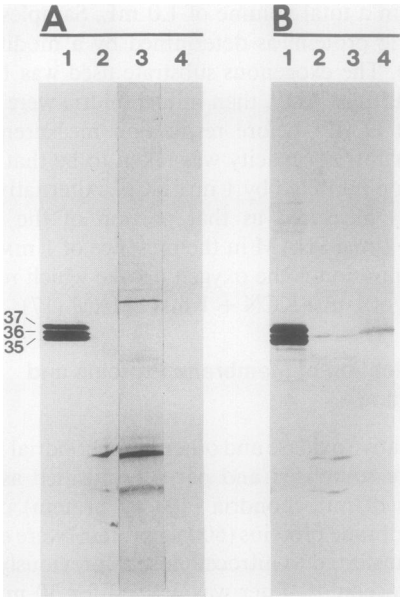


**Figure 2.** Immunoblots of potato alternative oxidase. Proteins were electrophoresed, transferred to nitrocellulose, and reacted with a monoclonal antibody against the *S. guttatum* alternative oxidase (8) as described in "Materials and Methods." Each panel includes lanes of *S. guttatum* mitochondria (A1, B1) for comparison, potato mitochondria (A2, B2), deoxy-BIGCHAP-insoluble mitochondrial membrane proteins from the pellet fraction (A3, B3), and deoxy-BIGCHAP-solubilized mitochondrial membrane proteins from the supernatant fraction (A4, B4). Panel A contains proteins from fresh potato tissue; panel B contains proteins from 24-h-aged potato discs. Mol wt in kD are shown to the left.

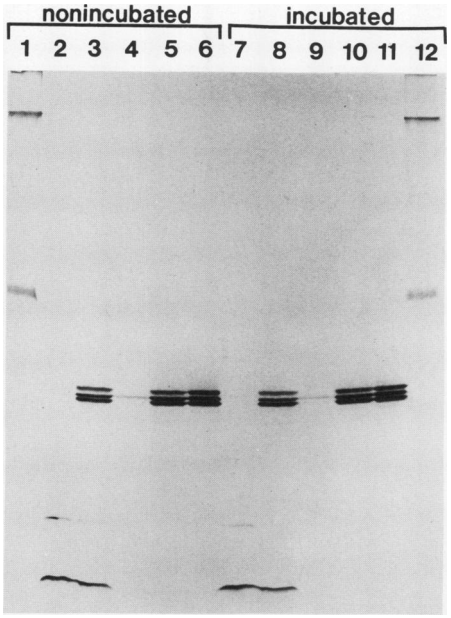


**Figure 3.** Mixing and incubation of mitochondrial samples. Samples 1 to 6 were boiled immediately upon addition of the mitochondria to the sample buffer. Samples 7 to 12 were incubated for 10 min at room temperature after addition of the mitochondria to the sample buffer and before boiling. Proteins were then electrophoresed, transferred to nitrocellulose, and reacted with a monoclonal antibody against the *S. guttatum* alternative oxidase (8). Lanes 1 and 12: mol wt standards; lane 2 and 7: fresh potato mitochondria; lanes 3 and 8: mixture of fresh potato and *S. guttatum* mitochondria; lanes 4 and 9: 24-h-aged potato disc mitochondria; lanes 5 and 10: mixture of 24-h-aged potato disc and *S. guttatum* mitochondria; lanes 6 and 11: *S. guttatum* mitochondria.

To further characterize the potato alternative oxidase, we separated the mitochondrial membrane polypeptides on two-dimensional gels (Fig. 4). Partially purified mitochondrial membrane proteins were isolated according to Elthon and McIntosh (6). Two potato membrane polypeptides are immunoreactive with the alternative oxidase antibody (panel D). These have the same apparent mol wt but different isoelectric points of approximately 7.1 and 6.7. They may therefore be different isoforms of the same protein. To visualize the potato alternative oxidase in immunoblots, high amounts of mitochondrial membrane proteins were loaded onto the gels. Consequently, many potato proteins are present in the India-ink stained blot (panel C) and we are unable to identify with certainty which are the immunoreactive ones. Since much less *S. guttatum* mitochondrial membrane protein need be loaded onto gels to visualize the alternative oxidase in immunoblots, the alternative oxidase proteins are readily identifiable on both the stained blot (panel A) and the immunoblot (panel B). They have an apparent mol wt of 37, 36, and 35 kD and isoelectric points of approximately 7.2 and 7.3.

## DISCUSSION

A fundamental question in plant bioenergetics concerns the mechanism(s) for governing the partition of electron flow between the Cyt pathway and the alternative pathway. Until recently there have been two major hindrances to answering this question: first, the methods for measurement of electron flow into either pathway; and second, the lack of a physical or molecular probe for the alternative oxidase. Measurements of Cyt and alternative pathway respiration are measurements of the capacities of the pathways obtained through inhibitor titration (reviewed in 17), not measurements of actual activities for the two pathways *in vivo*. Recently, differential fractionation of oxygen isotopes has been shown to be a viable method for the measurement of the engagement of the alternative and Cyt pathways (10). It is heartening, for those who do measure capacities, that engagement and capacities in these recent experiments were similar. There is one report for oxygen discrimination for potato tubers (18); however, as pointed out by Guy *et al.* (10), the value is probably strongly influenced by limited oxygen diffusion. Limited oxygen diffusion is still a major hurdle to overcome for many plant organs and tissues when one wishes to measure engagement (10). A molecular approach to the alternative pathway, such as the one described here which employed a monoclonal antibody, should allow a broader understanding of the mechanism of alternative pathway induction and complement knowledge gained from future experiments on the engagement of the electron transport pathways in plant mitochondria.

We have used a monoclonal antibody raised against the *S. guttatum* alternative oxidase (8) to follow the development of alternative oxidase capacity in aging potato slices. This antibody cross-reacts to the alternative oxidase of a variety of species including potato (8). Our results indicate that the levels of the 36 kD alternative oxidase protein parallel alternative pathway capacity in the mitochondria during the aging of potato slices. Increased alternative oxidase capacity in aged slice mitochondria appears to be the result of increased levels of the 36 kD protein present. Dizengremel and Lance (3) demonstrated the need for cytoplasmic (but not mitochondrial) protein synthesis to permit the increase in alternative pathway capacity in aging potato slice mitochondria. This suggests that new alternative oxidase proteins may be synthesized during the aging of potato slices, and that the gene(s) for alternative oxidase are nuclear-encoded in potato. The alternative oxidase genes were shown to be nuclear-encoded in *S. guttatum* (9, 11; D Rhoads, L McIntosh, unpublished results) and *Neurospora* (2, 16).

Respiration data have previously been interpreted to mean that the alternative pathway is present in whole tubers but destroyed by slicing and restored by aging (20, 28, 29). Our data suggest that another interpretation is possible: the active alternative oxidase protein (the 36 kD protein) is not present in significant amounts in whole tuber or fresh slice mitochondria but is synthesized *de novo* during aging of potato slices. Cyanide-resistant oxygen uptake previously measured in whole tubers (22) may have been nonmitochondrial in origin.

Dizengremel and Lance (4) reported variations in the protein composition of inner mitochondrial membranes with aging of potato slices. No increase in proteins of mol wt near 36 kD was reported (4); however, the alternative oxidase may not have been visible stained with Coomassie blue at the

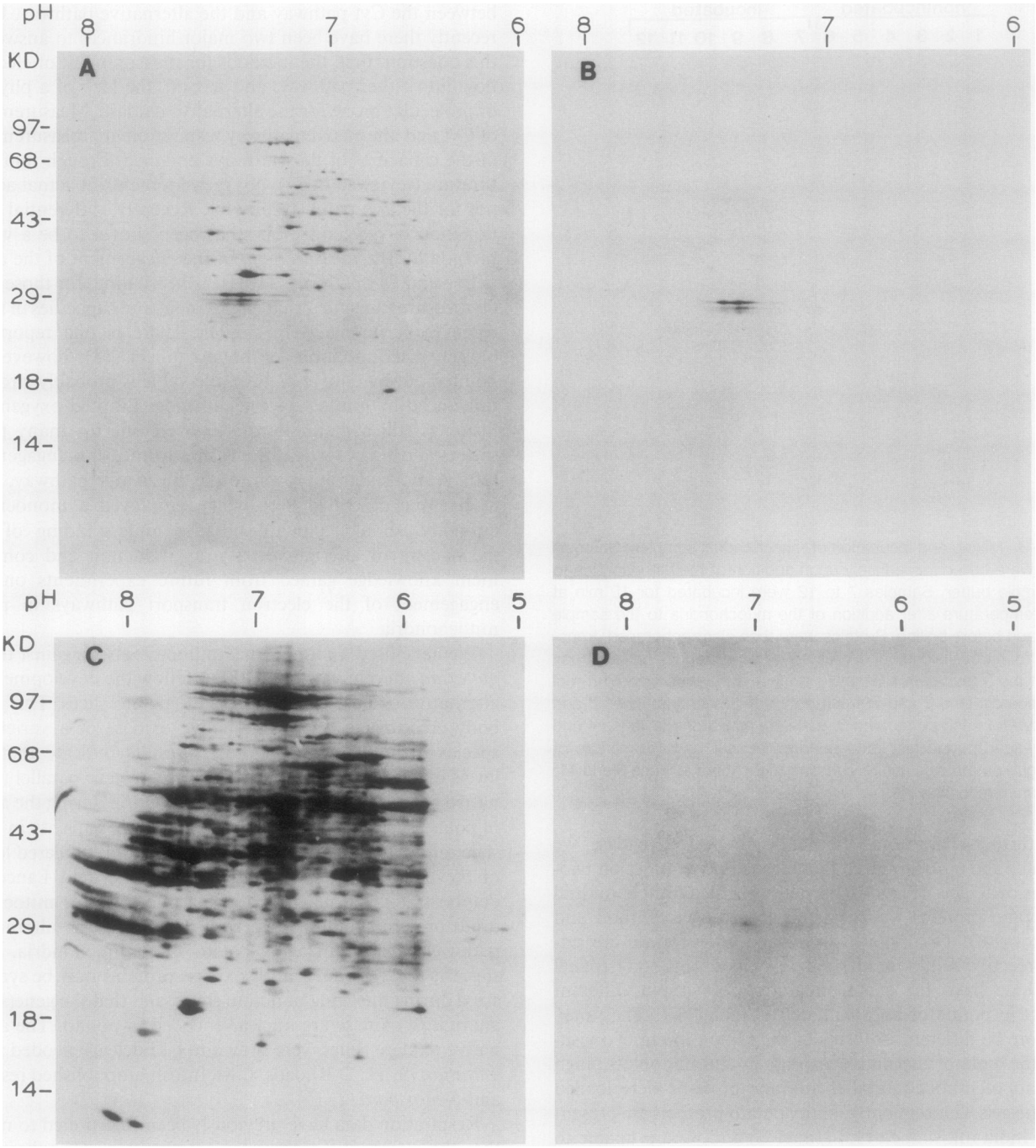


**Figure 4.** Two-dimensional gel electrophoresis and immunoblots of alternative oxidase proteins. Panels A and B are mitochondria membrane proteins from *S. guttatum*; panels C and D are mitochondrial membrane proteins from 24-h-aged potato discs. Panels B and D were reacted with a monoclonal antibody against the *S. guttatum* alternative oxidase (8). Panels A and C are the same blots as panels B and D, respectively, after staining for total protein with India ink.

concentrations of mitochondrial protein they loaded on their gels. Two-dimensional gel electrophoresis of the *in vitro* translation products of polysomal RNA from avocado fruits (32) and carrot roots (33) which were treated with ethylene and/or cyanide showed increases in proteins near the mol wt and pKas of potato and *S. guttatum* alternative oxidase proteins as determined in our laboratory (7, 8; D Rhoads, L McIntosh, unpublished results). Treatment with ethylene and/or cyanide has been shown to induce cyanide-resistant respiratory capacity in potato (1, 13, 24, 27, 31) and other storage organs (31). Dizengremel and Kader did report a decrease in the relative amounts of proteins with molecular weights less than 22 kD

(4). We have also found decreased levels of the 12 to 18 kD immunoreactive polypeptides in aged slice mitochondria.

Faint low mol wt bands similar to those seen in the immunoblots of fresh potato slice mitochondria (Fig. 2, lane A2) have also been seen in other higher plants (8; A Goyal, C Hiser, NE Tolbert, L McIntosh, unpublished data; RL Nickels and L McIntosh, unpublished data) and *Neurospora* strains which lack the higher mol wt alternative oxidase protein (16). There is presently no conclusive explanation for these bands.Our mixing experiment suggests that they are not the results of general degradory processes during sample preparation. It is still possible that they may be the products of proteases present (perhaps to a greater degree in fresh tissue than aged tissue) during mitochondrial isolation. They may be degradation products formed during the membrane breakdown which accompanies slicing (28, 30). Another possibility is that disruption of the membranes due to slicing does not allow for proper insertion of alternative pathway proteins into a complex and thus they are broken down. The low mol wt polypeptides may therefore result from turnover of noncomplexed alternative oxidase. If this were the case, then one could speculate that some level of alternative oxidase proteins are constitutively made but rapidly turned over unless some factor (induced by wounding and aging) stabilizes them.

We conclude that the potato alternative oxidase capacity increases at the same time as the 36 kD protein constituent rises in concentration in aging potato slice mitochondria. The levels of 2 to 3 cross-reactive, low mol wt polypeptides in the mitochondrial membrane concurrently decrease. It is unknown if the synthesis of potato alternative oxidase is transcriptionally and/or posttranscriptionally controlled, or if levels of the Cyt oxidase may influence the levels of the alternative oxidase. In *S. guttatum*, the relative levels of mRNA encoding the Cyt oxidase subunits I and II decrease during the time in which alternative oxidase capacity is increasing, raising the possibility that regulation of Cyt oxidase and alternative oxidase may be related (9). Experiments investigating the regulation of both Cyt oxidase and alternative oxidase on transcriptional and protein levels are currently being pursued.

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