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Respiration of Mitochondria Isolated from Leaves and Protoplasts of *Avena sativa*[1]

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BARBARA M. KELLY*[2] AND JOSEPH T. WISKICH
Botany Department, The University of Adelaide, Adelaide, South Australia, 5001

ABSTRACT

Mitochondria isolated from mesophyll protoplasts differed from mitochondria isolated directly from leaves of *Avena sativa* in that protoplast mitochondria (a) had a lower overall respiratory capacity, (b) were less able to use low concentrations of exogenous NADH, (c) did not respond rapidly or strongly to added NAD, (d) appeared to accumulate more oxaloacetate, and (e) oxidized both succinate and tetramethyl-*p*-phenylene-diamine (an electron donor for cytochrome oxidase) more slowly than did leaf mitochondria. It is concluded that cytochrome oxidase activity was inhibited, the external NADH dehydrogenase had a reduced affinity for NADH, succinate oxidation was inhibited, NAD and oxaloacetate porters were probably inhibited, and accessibility to respiratory paths may have been reduced in protoplast mitochondria. The results also suggest that there was a reduced affinity of a succinate porter for this substrate in oat mitochondria. In addition, all oat mitochondria required salicylhydroxamic acid (SHAM) as well as cyanide to block malate and succinate oxidation. Malate oxidation that did not appear to saturate the cytochrome pathway was sensitive to SHAM in the absence of cyanide, suggesting that the oat mitochondria studied had concomitant alternative and subsaturating cytochrome oxidase pathway activity.

Protoplasts are uniquely useful as models for cells. The absence of a cell wall allows ready access to the plasma membrane (22) and facilitates rapid cell fractionation (14). Protoplasts additionally provide the convenience of a homogeneous suspension (19). Oat protoplasts are being used to assess intracellular distribution of metabolites (14), regulation of ion and amino acid flux (19, 22), biosynthetic responses to pathogens (29), and the photosynthetic capacity of single cells (15). Consequently, the extent and nature of metabolic differences between protoplasts and cells must be assessed.

Comparisons between oat protoplasts and oat cells have not produced a consistent picture. Polyamine ratios differ (13) and protoplasts are less able to transport amino acids (28) than are cells. Nonetheless, photosynthetic capacities (16) and membrane potentials (27) are reported to be similar.

Because of the observation by one author, Kelly (18), that oat mesophyll protoplasts required high concentrations of O_2 for respiration and that their mitochondria were unable to oxidize succinate, we have examined the extent and cause of differences in the mitochondrial respiration of oat mesophyll protoplasts and oat leaves. We reduced the amount of O_2 required to measure maximal rates of protoplast respiration by better exclusion of air bubbles from the O_2 electrode chamber and showed osmotic stress experienced by protoplasts to be the probable primary cause of most other respiratory differences between protoplasts and leaves. The role of osmotic stress and the respiration of leaf segments and intact protoplasts will be presented subsequently. We report here on the comparative response of oat mesophyll protoplast and leaf mitochondria to single and multiple substrates, OAA, NAD, and respiratory inhibitors.

MATERIALS AND METHODS

Plant Material. *Avena sativa* cv Bulban seedlings were grown in vermiculite supplemented with half-strength Hoagland solution at 21°C under a cycle of 8 h of dark and 16 h of approximately 150 μmol/m^2·s white light. The ratio of incandescent to cool-white fluorescent light was greater than 0.2, providing far red light to enhance mitochondrial activity (24) and simulate the solar spectrum (2). Primary leaves were harvested 7 to 8 h after the beginning of the photoperiod 8 d after planting.

Preparation of Protoplasts. Protoplasts were isolated and purified as described by Rubinstein (27) with the modifications that the concentration of Cellulysin was 0.4% and the concentration of T20 dextran[3] and sorbitol in the gradient were 14% (w/w) and 0.44 M, respectively. A low level of incandescent light (about 15 w/m^2) was provided during digestion of the cell wall.

Purified protoplasts were centrifuged at 400*g* for 2.5 min in wash medium (1 mM Hepes, 2 mM Mes, 0.6 M sorbitol, 29 mM sucrose, 1 mM glucose, 1 mM $Ca[NO_3]_2$, 0.25 mM $MgCl_2$, 3 mM KCl, 1 mM KOH, and 1 mM NaOH, adjusted to pH 7.2 if necessary with KOH:NaOH 4:1) and resuspended in mitochondrial isolation medium (0.3 M mannitol, 30 mM MOPS, 10 mM isoascorbate, 1 mM EDTA, 0.2% [w/v] BSA, and 0.6% PVP [pH 7.5]).

When required, protoplasts were aged by leaving them in wash medium for 24 h at 4°C in the dark before transfer to mitochondrial isolation medium.

Preparation of Mitochondria. Mitochondria were isolated from protoplasts by gently homogenizing 7×10^7 protoplasts with a Teflon Potter-Elvehjem homogenizer (1 stroke) in 60 ml cold (4°C) mitochondrial isolation medium and from leaves by disrupting 50 g leaves with a polytron PTA-36 probe for 15 s in 200 ml cold mitochondrial isolation medium. The leaf brei was filtered through a 50 μm nylon mesh screen. Exposure to light was minimal throughout the isolation procedure.

The homogenate or filtrate was centrifuged at 500*g* for 15 min and recentrifuged at 6000*g*. Pellets from 500 and 6000*g* centrifugations were suspended in wash medium (0.4 M mannitol, 10 mM MOPS, 1 mM EDTA, and 0.2% [w/v] BSA [pH 7.2]), centrifuged at 250*g* for 10 min, and recentrifuged at 6000*g*. The 6000*g* pellet was suspended in about 1 ml suspension medium

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[3] Abbreviations: T20 dextran, dextran mol wt 20,000; MOPS, morpholinopropanesulfonic acid; TMPD, tetramethylphenylenediamine; SHAM, salicyl-hydroxamic acid; OAA, oxaloacetate.

(0.3 M mannitol, 0.2 M sucrose, 10 mM MOPS, 1 mM EDTA, and 0.1% [w/v] BSA [pH 7.2]).

Mitochondria were purified by a modification of the method of Day *et al.* (6) in that a discontinuous PVP gradient (PVP 0%, 3 ml; 2.3%, 5 ml, 5% 14 ml; 8.1%, 6 ml; 10%, 6 ml [top to bottom]) was used with 28% Percoll. The mitochondrial band was removed and washed three times in 70 ml suspension medium at 13,300*g*. The final pellet was suspended to about 1 mg protein/ml in suspension medium and stored at 0°C for 1 h before use.

Chemicals and Assays. O_2 uptake was measured at 25°C with a Clark-type O_2 electrode purchased from Hansatech Ltd. (Hardwick Industrial Estate, Norfolk, U.K.). The reaction medium contained 0.3 M mannitol, 6 mM $MgCl_2$, 15 mM KH_2PO_4, 0.1% (w/v) BSA, and known quantities of mitochondrial protein (about 0.1 mg/ml) (pH 7.2). The O_2 concentration of air-saturated medium was assumed to be 260 nmol/ml (17).

Protein was estimated by the method of Bradford (3) with the use of the prepared Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA).

Predominantly fluorescent light was measured with a quantum sensor (LiCor). Incandescent light was measured with a solar:meter (Kipp).

Chemicals were obtained from Sigma or Boehringer Mannheim (Sydney, Australia). Cellulysin was obtained from California Biochemicals.

Assessment of Data. Rates of O_2 uptake varied sufficiently during the year (Table I) to require experimentally induced changes in activity to be tabulated and assessed for significance as change per experiment.

RESULTS AND DISCUSSION

Substrate Oxidation. Mitochondria isolated from protoplasts oxidized TMPD, succinate, and low concentrations (3 mM) of exogenously supplied NADH more slowly than did mitochondria that had been isolated directly from leaves (Table I). The observed difference between protoplast and leaf mitochondria was irreversible, having been initiated before and maintained through 6 to 8 h of the same preparation and assay for both types of mitochondria.

Mitochondria were purified for most assays. Mitochondrial preparations from protoplasts were sometimes used without purification, since their Chl levels were much lower than those from leaves (mg protein/mg Chl: washed mitochondria from protoplasts = 5; washed mitochondria from leaves = 3). Cyt *c*-dependent O_2 uptake (6) indicated that both washed and purified mitochondria from protoplasts were 87% intact and that mitochondria purified from leaves were 92% intact. ADP/O ratios, RC values, and relative rates of oxidation of different substrates by washed mitochondria from protoplasts were similar to those of purified ones. Variability during the year (RC values and ADP/O ratios for malate oxidation ranged from over 4 to 1 and from 2 to below 1, respectively) was not dependent upon the source of mitochondria. There was little O_2 uptake in the absence of substrate, and it was rarely measured (at least five experiments each, nmol O_2 min^{-1} mg $protein^{-1}$: purified mitochondria from leaves, 20 ± 23; purified mitochondria from protoplasts, 10 ± 10; washed mitochondria from protoplasts, 3 ± 5). One typical experiment, demonstrating the response to some of the substrates tested, is shown for each type of mitochondria in Figure 1.

Protoplast mitochondria oxidized TMPD about half as fast as did mitochondria from leaves (Table I). TMPD is an artificial electron donor that donates electrons to Cyt *c* and *a* from the intermembrane space (11). Therefore, this result indicates some loss of Cyt oxidase activity in protoplast mitochondria.

Succinate (20–30 mM) and NADH (3–6 mM) were used in considerable excess (7). Succinate appeared to be marginally limiting for protoplast, and possibly leaf, mitochondria at a concentration of 10 mM, because an additional 10 mM succinate stimulated rates of O_2 uptake by 9 ± 5% (three experiments) for protoplast mitochondria and by 4 ± 3% (five experiments) for leaf mitochondria. Consequently, the affinity of a porter (9) or succinate dehydrogenase for succinate appeared to be low in oat mitochondria. Additional evidence that a porter was affected is provided later under "Multiple Substrates."

Raising initial succinate concentrations from 20 to 30 mM did not affect succinate/malate ratios. The increase in the average rate of oxidation of succinate for the experiments in Table IB reflects faster rates of oxidation of all substrates by the protoplast mitochondria, probably partly because of variation in seed lots. In spite of this increase, the average rate of succinate oxidation by protoplast mitochondria remained 30% lower than that of leaf mitochondria. Therefore, a reduced affinity of either porter or enzyme for succinate cannot fully explain the low rate of oxidation of this substrate by protoplast mitochondria.

The response of leaf mitochondria to added NADH as the assay progressed indicated that concentrations of 1.5 mM were saturating (data not shown). However, NADH concentrations of at least 6 mM were required for maximal rates of oxidation of NADH by protoplast mitochondria. Adding 3 mM NADH to protoplast mitochondrial suspensions that were oxidizing 3 mM NADH stimulated O_2 uptake by 18 ± 1% (three experiments). This results suggests a decreased affinity of the external dehydrogenase (11) for NADH in protoplast mitochondria.

In addition, protoplast mitochondrial ability to use even high concentrations of NADH may have been impaired. The differ-

Table I. *Substrate Oxidation by Protoplast and Leaf Mitochondria*

Purified mitochondria were prepared and assayed as described in "Materials and Methods." Value given is the average ± SD of the state 3 rates of substrate oxidation. A, in each experiment the five substrates were assayed as follows: succinate, 20 mM + ATP; NADH, 3 mM; TMPD, 2 mM + 10 mM ascorbate; glycine, 33 mM; malate, 30 mM + 30 mM glutamate. B, as A except succinate, 30 mM + 150 μM ATP; NADH, 6 mM. *n*, number of experiments.

Source of Mitochondria	Succinate	NADH	TMPD	Glycine	Malate	*n*
	nmol O_2 min^{-1} mg $protein^{-1}$					
Leaves						
A	205 ± 37	325 ± 84	803 ± 101	241 ± 24	223 ± 56	4
B	297 ± 57	335 ± 73	860 ± 240	278 ± 93	301 ± 61	10
Protoplasts						
A	91 ± 24	155 ± 49	288 ± 110	149 ± 40	145 ± 29	8
B	206 ± 26	277 ± 99	465 ± 134	348 ± 80	404 ± 86	3
Aged protoplasts						
A	52 ± 12	83 ± 9	148 ± 12	50 ± 12	48 ± 2	3

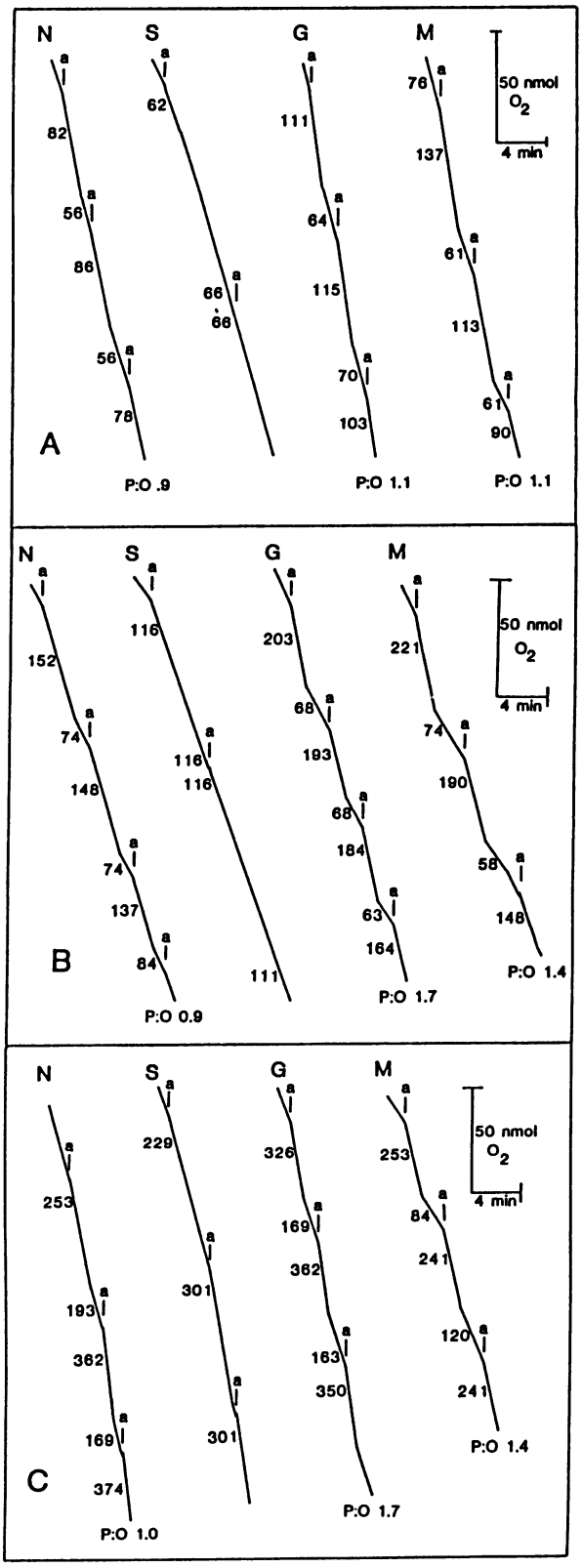

FIG. 1. Substrate oxidation was initiated by addition to 0.5 ml reaction medium of 3 mM NADH (N), 20 mM succinate + 150 μM ATP (S), 33 mM glycine (G), or 30 mM malate + 30 mM glutamate (M) plus A, 0.12 mg protein of washed mitochondria from protoplasts; B, 0.065 mg protein of purified mitochondria from protoplasts; or C, 0.055 mg protein of purified mitochondria from leaves. ADP addition, 100 μM at (a); ADP/O ratio (P:O). Rates are expressed as nmol O_2 min^{-1} mg $protein^{-1}$.

ence in rates of oxidation of 6 mM NADH by protoplast and leaf mitochondria was not significant (Table IB). However, rates of oxidation of 6 mM NADH, when compared to rates of oxidation of malate (NADH/malate ratio), remained below those of leaves.

Adding malate during the assay stimulated the rate of oxidation of malate by all mitochondria, probably due to the unfavorable malate-OAA equilibrium (30). Glycine was used at a concentration (33 mM) that was saturating for both protoplast and leaf mitochondria (data not shown). Protoplast and leaf mitochondria did not differ significantly in their ability to oxidize malate (Table I). An apparent inhibition of glycine oxidation by protoplast mitochondria in one series of experiments (Table IA) was not supported by statistically significant lower normalized rates (glycine/malate ratios).

The osmotic potential of the medium used to suspend the protoplasts was about −1.8 MPa. Withholding water from corn seedlings long enough to induce similar levels of osmotic stress markedly and irreversibly affected their mitochondria, apparently due to alterations in mitochondrial membranes (20). The inhibition of respiration in corn seedling mitochondria (1, 20) differed from that of oat protoplast mitochondria in that malate, succinate, and external NADH were equally affected in the former.

Aging oat protoplasts for 24 h before isolating the mitochondria affected the pattern of mitochondrial substrate oxidation. When mitochondria had been isolated from aged protoplasts, oxidation of malate, succinate, and NADH was equally inhibited, as was reported (1) for mitochondria isolated from stressed corn. In addition to the change in pattern of inhibition of substrate oxidation, the inhibition was more severe (Table I). Therefore, the inhibition of substrate oxidation in mitochondria from freshly prepared protoplasts, which appeared to particularly affect Cyt oxidase, succinate dehydrogenase, and the external NADH dehydrogenase, may reflect an early stage in the response of mitochondrial membranes to the effect of osmotic stress on cells.

The Effect of Added NAD. The requirement for added substrate to take up O_2 (see above) suggests that mitochondria isolated from protoplasts had low levels of oxidizable substrate. Nonetheless, the contraced, electron-dense matrix observed in mitochondria in protoplasts (26) is consistent with high concentrations of some matrix constituents. Limited amounts of oat protoplast mitochondrial material prevented direct measurement of matrix NAD levels. We used the response of malate and glycine oxidation to added NAD to estimate the relative sufficiency of matrix NAD in protoplast and leaf mitochondria. If matrix NAD is suboptimal, adding NAD to the reaction medium stimulates oxidation of malate and glycine (12), prevents progressive retardation of glycine oxidation (as will be shown), and permits generation of enough NADH to increase the activity of the rotenone-insensitive bypass (4) and to reduce progressive retardation of malate oxidation due to increased OAA levels (4, 30).

Malate oxidation by leaf mitochondria was stimulated by exogenous NAD and inhibited by rotenone (Table II.) Added NAD partially alleviated the rotenone-induced inhibition. It can be concluded that leaf mitochondria took up exogenous NAD readily and usually had suboptimal levels of NAD (the internal NAD concentration was too low to fully activate the rotenone-insensitive bypass).

Added NAD did not stimulate malate oxidation by mitochondria from protoplasts unless the protoplasts had been aged for 24 h (Table II). Aging protoplasts markedly reduced the ability of their mitochondria to oxidize malate. Rotenone inhibited malate oxidation by washed mitochondria from aged protoplasts, although the rate of O_2 consumption (Table II) was so low that it approached that of residual (21) respiration (20 nmol O_2 min^{-1} mg $protein^{-1}$, one experiment). Adding NAD totally alleviated

Table II. *Response of Leaf and Protoplast Mitochondria to Rotenone and Added NAD*

Mitochondria were prepared from protoplasts (fresh or aged for 24 h) or from leaves. Values: first column, average state 3 rate of oxidation ± SD of 30 mM malate + 30 mM glutamate with no addition; columns 2 to 4, average change per experiment ± SD in state 3 rate upon addition of 1 mM NAD, 20 μM rotenone, or both. Mitochondria were either purified (p) or washed (w). Number of experiments are shown in parentheses.

Source of Mitochondria	Purity	No addition	NAD	Rotenone	
		nmol O_2 min^{-1} mg protein^{-1}	*% stimulation*	*% inhibition without NAD*	*% inhibition with NAD*
Leaves	p	279 ± 73 (14)	38 ± 17 (14)	47 ± 11 (5)	18 ± 10 (4)
Protoplasts					
Fresh	p	247 ± 148 (7)	2 ± 5 (5)	26 ± 19 (5)	26 ± 19 (5)
Aged	p	48 ± 1 (3)	47 ± 10 (3)		
	w	40 ± 7 (3)	73 ± 39 (3)	13 ± 5 (3)	0 ± 0 (3)

rotenone-induced inhibition of O_2 uptake by mitochondria from aged protoplasts (Table II). Therefore, mitochondria from aged protoplasts took up NAD readily, apparently in response to low internal NAD levels.

Mitochondria from fresh protoplasts did not respond obviously to exogenous NAD. The faster rates of oxidation of malate and glycine by mitochondria from fresh as compared with aged protoplasts (Table I) are consistent with higher matrix NAD levels in mitochondria from fresh protoplasts. Nonetheless, NAD levels of mitochondria from fresh protoplasts were low enough to permit inhibition by rotenone (Table II). The failure of mitochondria from freshly prepared protoplasts to respond to added NAD even after inhibition by rotenone is consistent with reduced NAD transport into these mitochondria.

Adding NAD to mitochondrial suspensions that were oxidizing glycine provided additional evidence that was consistent with retarded NAD transport into mitochondria from fresh protoplasts. Rates of glycine oxidation decreased over time (Fig. 2, A and D). Adding NAD immediately alleviated the decrease and stimulated state 3 and 4 oxidation of glycine by leaf mitochondria (Fig. 2, D and E). Added NAD did not immediately stimulate glycine oxidation by protoplast mitochondria, even after rates had decreased markedly (Fig. 2, B and C). There was, however, some abatement of the retardation of the rate of oxidation of glycine by protoplast mitochondria a few minutes after the addition of NAD (Fig. 2, A and B), suggesting slow uptake of the cofactor.

The NAD porter mediates nearly all flux of NAD into and out of the matrix, the influx being energy-dependent (25). The evidence presented is consistent with relatively high protoplast mitochondrial matrix NAD levels (possibly maintained by inhibited NAD efflux) and reduced energy-dependent NAD influx, both of which were reversed by aging the protoplasts. The results can be explained by an inhibition of the protoplast mitochondrial porter, the effects of which were diminished by changes in membrane integrity, by a gradual diminution of matrix NAD levels, or by a change in the protein itself.

Apparent Relative OAA Levels. Rates of oxidation of malate by protoplast mitochondria decreased more markedly over time than did those of leaf mitochondria (Fig. 1). This decrease in rate could be temporarily alleviated by holding the protoplast mitochondria in state 4 (without added ADP) (Fig. 3A). Withholding ADP allows NADH to accumulate (10). Increases in rates of oxidation of malate during and immediately after prolonged state 4 conditions are attributed, in part, to a shift in the OAA-malate equilibrium due to reduction of OAA by the accumulated NADH (4, 10). Therefore, the decrease in rates of oxidation of malate over time is consistent with OAA accumulation in protoplast mitochondria, in spite of the presence of glutamate.

Mitochondria from leaves appeared to accumulate less OAA than did mitochondria from protoplasts. The decrease in rate of malate oxidation by leaf mitochondria over time could be totally alleviated by exogenous NAD (Fig. 3B). However, withholding ADP had no effect (Fig. 3C). Therefore, the limiting factor for

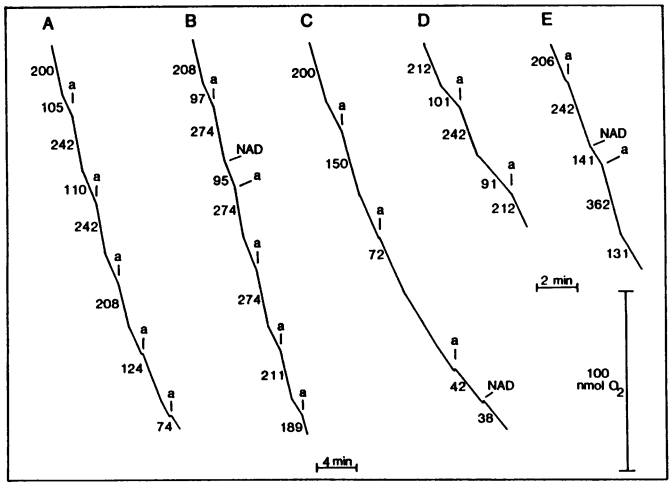

FIG. 2. Effect of added NAD on oxidation of glycine by purified mitochondria from protoplasts (A, B, C) or leaves (D, E). Control for B, A; for E, D. Assay initiated by addition of 33 mM glycine, 100 μM ADP, and about 0.05 mg mitochondrial protein to 0.5 ml reaction medium. Assay initiated 1.5 min prior to period shown (C). Additions: ADP, 100 μM at (a); NAD, 1 mM. Rates are expressed as nmol O_2 min^{-1} mg protein^{-1}.

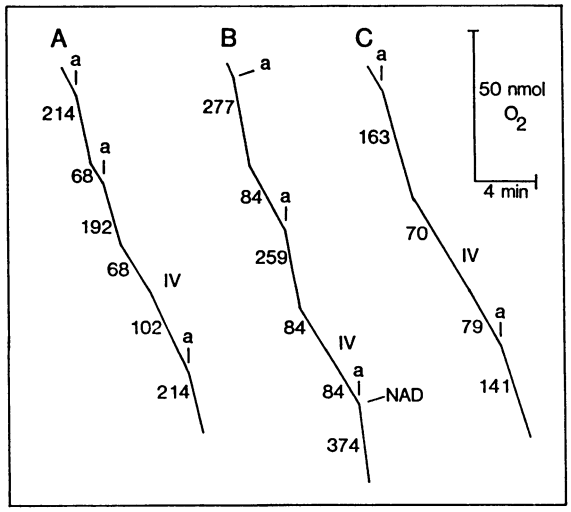

FIG. 3. Effect of withholding ADP on subsequent rates of oxidation of 30 mM malate + 30 mM glutamate by purified mitochondria from protoplasts (A) or leaves (B, C). Assay initiated by addition of substrate and about 0.05 mg mitochondrial protein to 0.5 ml reaction medium. Prolonged state 4 (IV). Additions: ADP, 100 μM at (a); NAD, 1 mM. Rates are expressed as nmol O_2 min^{-1} mg protein^{-1}.

Table III. *Inhibition of Leaf and Protoplast Mitochondrial Respiration by KCN and SHAM*

Comparison of the state 3 rates of substrate oxidation by purified mitochondria after adding either 2 mM KCN or 2 mM SHAM or both. A, Average uninhibited state 3 rate of substrate oxidation ± SD for experiments given; B, average inhibition of state 3 rate of oxidation per experiment ± SD. S, 20–30 mM succinate + 150 μM ATP; M, 30 mM malate + 30 mM glutamate; N, 3–6 mM NADH.

	Leaf Mitochondria			Protoplast Mitochondria		
	S	M	N	S	M	N
A	*nmol O_2 min^{-1} mg $protein^{-1}$*					
	203 ± 71 (9)	204 ± 86 (10)	298 ± 111 (5)	112 ± 59 (7)	196 ± 100 (7)	116 ± 68 (7)
B	*% inhibition*					
CN	62 ± 16 (3)	61 ± 10 (5)	83 ± 3 (3)	44 ± 18 (3)	47 ± 11 (3)	82 ± 4 (5)
SHAM	17 ± 8 (4)	35 ± 9 (8)	22 ± 1 (2)	25 ± 8 (6)	39 ± 6 (6)	19 ± 6 (3)
CN + SHAM	75 ± 10 (5)	80 ± 6 (5)	88 ± 4 (3)	71 ± 10 (5)	77 ± 11 (6)	90 (1)

Table IV. *Response of Protoplast and Leaf Mitocondria to a Second Substrate*

Values are: A, the average increase per experiment ± SD in rates of O_2 consumption by purified mitochondria from leaves (LF) or washed mitochondria from protoplasts (PR) upon addition of a second substrate: B, the average rate of oxidation of the initial substrate ± SD for these experiments. Substrates as in Table I.

Added Substrate	Initial Substrate			
	Succinate	Malate	NADH	Glycine
A	*% stimulation*			
Succinate				
LF		21 ± 14 (6)	88 ± 34 (5)	54 ± 20 (6)
PR		0 ± 0 (5)	30 ± 13 (4)	8 ± 5 (5)
Malate				
LF	21 ± 26 (6)		47 ± 12 (3)	65 ± 18 (4)
PR	118 ± 48 (4)		39 ± 8 (4)	28 ± 12 (6)
NADH				
LF	122 ± 24 (5)	75 ± 49 (5)		52 ± 36 (6)
PR	189 ± 128 (3)	17 ± 12 (5)		20 ± 15 (6)
Glycine				
LF	107 ± 43 (4)	64 ± 20 (3)	42 ± 18 (7)	
PR	133 ± 13 (3)	17 ± 5 (5)	14 ± 17 (6)	
B	*nmol O_2 min^{-1} mg $protein^{-1}$*			
LF	222 ± 81 (7)	235 ± 96 (7)	282 ± 98 (7)	300 ± 97 (6)
PR	37 ± 14 (5)	94 ± 25 (6)	70 ± 14 (8)	99 ± 46 (6)

malate oxidation by leaf mitochondria appeared to be the amount of reducible pyridine nucleotide rather than the proportion of reduced nucleotide available to reduce OAA.

In addition, protoplast mitochondria were less affected by exogenous OAA than were leaf mitochondria. OAA (0.15 mM) inhibited malate oxidation by leaf mitochondria by 40 ± 14% and by protoplast mitochondria by 16 ± 1% (two experiments each). Adding 30 mM glutamate alleviated the inhibition by 32% for leaf mitochondria and 41% for protoplast mitochondria (two experiments each) indicating that glutamate oxaloacetate transaminase (4) was equally active in reducing OAA levels in protoplast and leaf mitochondria.

Both efflux and influx of OAA are mediated by a porter (8). Therefore, the data are consistent with some inhibition of the OAA porter in protoplast mitochondria and consequent greater retention of OAA by these mitochondria.

Response to KCN and SHAM. KCN and SHAM were used to test for a difference in the relative activity of the Cyt oxidase and alternative pathways (21) in protoplast as compared with leaf mitochondria. Protoplast mitochondria were slightly more resistant to KCN. However, the two types of mitochondria did not differ significantly in their response to 2 mM KCN and to 2 mM SHAM (Table III).

Oxidation of exogenously supplied NADH usually requires only Cyt oxidase pathway activity (21, 23). Indeed, with NADH as substrate, over 80% of the O_2 uptake was sensitive to KCN (Table III) or 20 μM antimycin (83% inhibition, one experiment, protoplast mitochondria). There was a low, probably nonspecific decrease in the rate of O_2 consumption when SHAM was used alone (Table III).

With malate or succinate as substrate, only about half of the O_2 uptake could be prevented by adding KCN. Antimycin inhibited protoplast malate oxidation by 40 ± 11% (three experiments) and succinate oxidation by 45 ± 5% (two experiments). The addition of SHAM was required to maximally inhibit malate or succinate oxidation by either protoplast or leaf mitochondria (Table III), indicating good access of electrons from both succinate and malate to the alternative pathway when the Cyt oxidase pathway was blocked or saturated.

Succinate oxidation was no more sensitive to SHAM, used alone, than was oxidation of NADH (Table III). Malate oxidation, however, was quite sensitive to SHAM used alone, indicating concomitant alternative and Cyt oxidase pathway activity.

The sensitivity of malate oxidation to SHAM was observed through the year with several seed lots and did not correlate well with poor coupling. The apparently constant concomitant oxidation of malate by Cyt oxidase and alternative pathways was not consistent with overflow from a saturated Cyt oxidase pathway. Leaf mitochondria oxidized NADH more rapidly than, and succinate as rapidly as, malate. Yet our evidence indicates that exogenous NADH was oxidized exclusively by the Cyt oxidase pathway and that succinate was not oxidized by the alternative pathway unless the Cyt oxidase pathway was blocked (Table III). Apparent concomitant alternative and subsaturating Cyt oxidase pathway activity has also been observed in soybean mitochondria (5).

Multiple Substrates. The response to a second substrate provided evidence that the overall respiratory capacity of leaf mitochondria was considerably higher than that of protoplast mitochondria. Leaf mitochondria not only oxidized most single substrates more rapidly than did protoplast mitochondria (Table I) but were also more responsive to the addition of a second substrate (Table IV). Succinate was oxidized so slowly by protoplast mitochondria that rates of O_2 uptake increased markedly upon addition of another substrate. With all other substrates, there was a greater increase in rates of O_2 uptake upon addition of a second substrate to leaf than to protoplast mitochondria. These results indicate a greater respiratory capacity for leaf than for protoplast mitochondria.

The difference in relative rates of oxidation of succinate and malate by protoplast and leaf mitochondria made apparent a restricted response of oat mitochondria to these two substrates. Adding malate to suspensions oxidizing succinate, or vice versa, did not increase rates of respiration beyond that of the single

most rapidly oxidized of these two substrates (Table IV). However, either malate or succinate as second substrate increased rates of O_2 uptake by mitochondria that were oxidizing other substrates. For example, leaf mitochondria, which oxidized succinate and malate at about the same rate (Table IV, B), showed little change in rates of O_2 uptake upon addition of either succinate or malate to suspensions that were oxidizing the other substrate (Table IV, A). However, adding either succinate or malate to leaf mitochondria that were oxidizing NADH markedly increased rates of O_2 consumption.

The results suggest competition between succinate and malate. Our results are more consistent with competition for transport into the mitochondria than for competition for respiratory pathways subsequent to complexes I and II. Electrons from malate and succinate did not appear to rely equally upon alternative and Cyt oxidase pathways (Table III). Further, rates of O_2 consumption increased slightly (20 ± 10%, four experiments) when succinate was added to protoplast mitochondria that were oxidizing malate in the presence of SHAM. Thus, electrons from succinate may have had access to some different Cyt oxidase pathways than did those from malate. Competition between succinate and malate for complexes III and IV is not precluded, but the results are more consistent with a competition between these substrates for limited transport into oat mitochondria.

ADP/O ratios and the degree of coupling of mitochondrial suspensions that were presented with both malate and succinate were more characteristic of malate than succinate oxidation (data not shown). Therefore, it is likely that succinate competed weakly with malate for transport into the mitochondria. The response of protoplast mitochondria, in particular, to different concentrations of succinate was consistent with reduced transport of this substrate.

CONCLUSIONS

Oat mitochondria had considerable alternative pathway activity. Alternative pathway activity appeared to be concomitant with subsaturating Cyt oxidase pathway activity during malate, but not during succinate, oxidation.

There is evidence for a rather low affinity of a porter for succinate in protoplast, and possibly also in leaf, mitochondria.

Mitochondria that were isolated from protoplasts differed from those that had been taken directly from leaves in a number of ways. In protoplast mitochondria (a) the respiratory capacity was lower than that of leaf mitochondria as indicated by the response to multiple substrates, (b) succinate oxidation was inhibited, as succinate was oxidized much more slowly than any other substrate and more slowly than this substrate was oxidized by leaf mitochondria, (c) external NADH dehydrogenase had a reduced affinity for NADH, because protoplast mitochondria were less able to use low concentrations of NADH than were leaf mitochondria, (d) Cyt oxidase activity was inhibited, since TMPD oxidation was markedly retarded, (e) the NAD porter appeared to be inhibited, since mitochondria from fresh protoplasts could not respond rapidly or strongly to NAD, although they were sensitive to rotenone, and (f) there was evidence for inhibition of the OAA porter, because protoplast mitochondria responded weakly to exogenous OAA and appeared to accumulate more OAA than did leaf mitochondria.

The observed differences between protoplast and leaf mitochondria are entirely consistent with a change in protoplast mitochondrial membranes that affected the activity of some constituent porters and enzymes and, possibly, the accessibility to respiratory paths.

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