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Orientation of Electron Transport Activities in the Membrane of Intact Glyoxysomes Isolated from Castor Bean Endosperm[1]

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ABSTRACT

Intact glyoxysomes were isolated from castor bean endosperm on isometric Percoll gradients. The matrix enzyme, malate dehydrogenase, was 80% latent in the intact glyoxysomes. NADH:ferricyanide and NADH:cytochrome *c* reductase activities were measured in intact and deliberately broken organelles. The latencies of these redox activities were found to be about half the malate dehydrogenase latency. Incubation of intact organelles with trypsin eliminated NADH:cytochrome *c* reductase activity, but did not affect NADH:ferricyanide reductase activity. NADH oxidase and transhydrogenase activities were negligible in isolated glyoxysomes. Mersalyl and Cibacron blue 3GA were potent inhibitors of NADH:cytochrome *c* reductase. Quinacrine, Ca^{2+}, and Mg^{2+} stimulated NADH:cytochrome *c* reductase activity in intact glyoxysomes. The data suggest that some electron donor sites are on the matrix side and some electron acceptor sites are on the cytosolic side of the membrane.

Glyoxysomes isolated from endosperm of germinating castor bean seedlings contain electron transport activities which are distinguishable from those of the ER (13). The enzymes, measured as NADH:CCR[3] and NADH:FCR, appear to be integral proteins of the glyoxysomal membrane, as the activities remain in membranes following treatment with 0.1 M Na_2CO_3 (11). This treatment has been demonstrated to effectively separate matrix from membrane proteins in peroxisomes (12) and glyoxysomes (11). Glyoxysomal palmitoyl-CoA oxidation can be coupled to electron transport reactions *in vitro* by reconstitution of carbonate-washed membranes with matrix fractions (11). The components of this electron transport system may thus be responsible for the transport of reducing equivalents, generated by intraglyoxysomal metabolism, across the glyoxysomal membrane to external acceptors. The glyoxysomal system may be analogous to that found in mammalian liver microsomes, consisting of a flavoprotein NADH reductase (measured as NADH:FCR), which transfers electrons to Cyt b_5 (measured as NADH:CCR) (10).

The purpose of this investigation was to determine the orientation of the electron transport functions and the direction of electron flow with respect to the matrix and cytosolic sides of the glyoxysomal membrane. Two main approaches were utilized to examine the orientation of the redox activities in isolated intact glyoxysomes, enzyme latencies (stimulation of activity upon organelle breakage) and treatment with protease. MDH displays a high degree of latency in glyoxysomal preparations when measured in isoosmotic conditions (8). Thus, MDH latency is a useful measure of the integrity of the glyoxysomal membrane. We have compared the latency of MDH with the latencies of redox enzyme activities to obtain information on the location of the electron donor and acceptor sites of the membrane redox enzymes. Trypsin treatment of intact glyoxysomes was utilized to probe the exposure of reductase activities at the cytosolic membrane face. We have also used some known effectors of other membrane electron transport systems to ascertain the nature of the components of the glyoxysomal system.

In order to obtain preparations of intact glyoxysomes, it was first necessary to develop an isolation procedure which would minimize changes in the osmotic environment of the organelles. Sucrose gradients were unsuitable as the organelles band at high osmolarities and subsequent dilution for centrifugation or assay invariably damages these osmotically fragile organelles. We have therefore developed an isolation procedure similar to that used by Mettler and Beevers (17) utilizing rapid centrifugation on isoosmotic Percoll gradients which results in stable preparations of intact glyoxysomes.

MATERIALS AND METHODS

Plant Material. Seeds of Castor bean (*Ricinus communis* L.) were surface sterilized in 0.02% (w/v) hypochlorite for 15 min, rinsed thoroughly in tap water, and germinated in moist vermiculite in the dark for 4.5 d at 30° C. Endosperm was separated from cotyledons, rinsed once with about 500 ml ice-cold distilled water, and kept chilled on ice prior to homogenization.

Chemicals. Percoll was obtained from either Pharmacia or Sigma. Cibacron blue 3GA was from Sigma. All other chemicals and biochemicals used in experiments were reagent grade, stored under manufacturer's suggested conditions.

Isolation of Glyoxysomes on Percoll gradients. Endosperm was chopped on ice with multiple razor blades to obtain 4 to 5 mm pieces for 2 min in one volume of grinding medium consisting of 13% (w/w) sucrose, 150 mM Tricine-KOH (pH 8.0), 10 mM KCl, 1 mM EDTA, and 1 mM $MgCl_2$ and subsequently ground with a mortar and pestle on ice for about 5 min to a thin paste. The resulting brei was filtered through nylon cloth and centrifuged at 970*g* for 10 min. Following separation from the floating lipid pad and nuclear pellet, 5 to 8 ml of supernatant from this 970*g* spin was carefully layered upon a 28 ml self-generated Percoll gradient of 20 to 50% (v/v) Percoll containing 0.5 M sucrose, 25 mM Tricine-KOH (pH 7.5), and 0.1% BSA (w/v) (gradient buffer). Percoll stock was dialyzed for 24 h against 100 volumes of distilled H_2O. Percoll gradients were generated

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[3] Abbreviations: NADH:CCR, NADH:Cyt *c* reductase; NADH:FCR, NADH-ferricyanide reductase; MDH, malate dehydrogenase; ICL, isocitrate lyase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

immediately prior to loading by centrifuging 33 ml of 30% (v/v) Percoll containing gradient buffer at 30,500*g* for 15 min in a TV-850 vertical rotor (screw caps) using a Sorvall OTD-50 ultracentrifuge. The upper 1.5 to 2 cm of the gradient were then removed to allow for sample loading. Loaded gradients were centrifuged in the vertical rotor at 18,100*g* for 12 min. Gradients were then either fractionated into 1.5 ml fractions using an ISCO density gradient fractionator, or previously identified glyoxysomal bands were carefully aspirated from gradients with a syringe and pooled.

Percoll Removal by Chromatography on Sepharose 2B. Glyoxysomes obtained directly from Percoll gradients were applied to a 2.5 × 15 cm column of Sepharose 2B previously equilibrated with 0.5 M sucrose, 10 mM Hepes-KOH (pH 7.5), and 0.01% (w/v) BSA (column buffer). The column was then eluted with column buffer, and the eluate absorbance was monitored at 254 nm. Fractions containing peak MDH activity were pooled and pelleted at about 2,000 to 8,000*g* until a loose pellet was obtained and very gently resuspended with a Pasteur pipet in column buffer to produce a suspension of glyoxysomes containing about 0.5 to 1.0 mg protein ml^{-1}. For experiments involving trypsin treatment of intact organelles, dextran (average mol wt 500,000) was substituted for BSA in the column buffer.

Enzyme Assays. Assays using both intact and broken glyoxysomes were conducted in column buffer, and all reagents were prepared in column buffer and adjusted to pH 7.5 with KOH or HCl. Glyoxysomes were broken by dilution with 2 volumes of 0.225 M KCl. MDH was assayed in the direction of malate formation using 100 μM NADH and 1 mM oxalacetate. NADH:FCR, NADH:CCR, and NADPH:CCR were assayed by the method of Hicks and Donaldson (13). NADP:6-P-gluconate dehydrogenase (21), fumarase and ICL (4), CDP-choline:diacylglyceride-phosphorylcholine transferase (15), and Cyt *c* oxidase (18) were assayed as described. Transhydrogenase activities using the analogs, 3-acetyl-NAD and 3-acetyl-NADH were assayed at 375 nm, assuming an extinction coefficient of 5.1 mM^{-1} (14). NADPH:CCR, Cyt *c* oxidase and CDP-choline:diacylglyceride-phosphorylcholine transferase were also assayed in mitochondrial and ER fractions isolated on sucrose gradients (11).

Protein was assayed by the method of Vincent and Nadeau (26) in Percoll-containing gradient fractions or Bradford (2) in suspensions of Percoll-free glyoxysomes.

RESULTS

Isolation of Glyoxysomes. The distribution of marker enzymes following separation of castor bean endosperm homogenate on self-generated 20 to 50% Percoll gradients is shown in Figure 1. Glyoxysomes, identified by ICL activity, were well separated from fumarase, the mitochondrial marker. NADP:6-P-gluconate dehydrogenase, which is present in both the proplastids and cytosol in germinating castor bean endosperm (21), did not sediment into the gradient.

Percoll has been reported to damage leaf peroxisomes over extended periods of incubation (20). To separate the organelles from the Percoll centrifugation medium, glyoxysomal fractions aspirated from Percoll gradients were chromatographed on Sepharose 2-B as suggested by Schmitt and Edwards (20). Glyoxysomes were eluted at the void volume, while Percoll, as assayed qualitatively by precipitation with Bradford protein reagent or trichloroacetic acid, typically eluted in a broad peak immediately following (data not shown). Centrifugation and gentle resuspension of the Percoll-free organelles resulted in a preparation of glyoxysomes which maintained membrane integrity (see below) for several hours on ice. Yields of organelles from Percoll gradients were typically about 0.05 mg glyoxysomal protein (g endosperm fresh weight)$^{-1}$, which is considerably less than for comparable sucrose gradients. The procedure produced intact organelles (80% MDH latency or higher, see below) in about 25% of all trials.

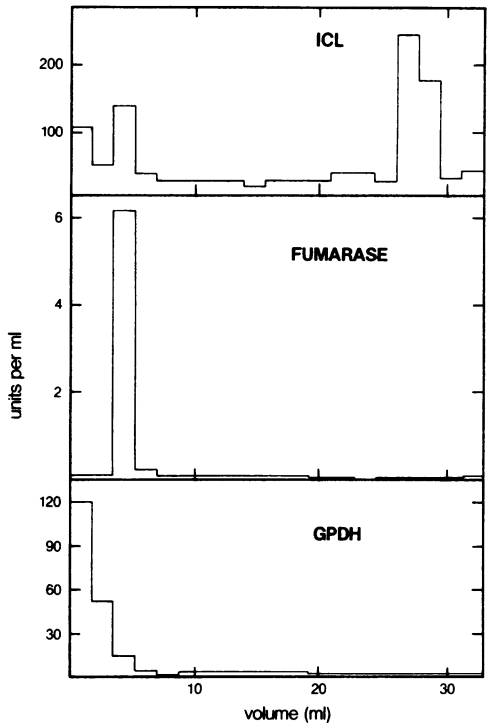

FIG. 1. Distribution of marker enzymes for glyoxysomes (ICL), mitochondria (fumarase) and proplastids (GPDH, 6-P-gluconate dehydrogenase) following centrifugation of castor bean homogenate on self-generated 20 to 50% (v/v) Percoll gradient. Units are nmol/min except fumarase, μmol/min.

Table I. *Comparison of Specific Activities of Marker Enzymes for Mitochondria and ER Measured in Glyoxysomes Isolated from Percoll Gradients versus Sucrose Gradient-Isolated Mitochondria and ER*

Specific activities for mitochondria and ER fractions are from Fang *et al.* (11). (PC transferase—CDP-choline:diacylglyceride phosphocholine transferase).

Enzyme	Glyoxysomes	Mitochondria	ER
	units·mg^{-1} protein		
Cyt *c* oxidase[a]	45	5241	38
NADPH:CCR[a]	4	9	132
PC transferase[b]	31	20	526

[a] nmol/min. [b] pmol/min.

It is evident from Figure 1 that glyoxysomes isolated on Percoll gradients were separated from plastids and mitochondria. However, contamination by ER and mitochondria in glyoxysomes could not be accurately estimated in fractions obtained from Percoll gradients. Mitochondria and ER co-migrated to the interface of the gradient and were not well separated from the cytosolic fractions (data not shown). We therefore have summarized in Table I mitochondrial and ER marker enzyme specific activities measured in Percoll-isolated glyoxysomes compared with those from mitochondria and ER isolated on sucrose gradients. The data in Table I confirm the low degree of ER and mitochondrial cross-contamination measured in glyoxysomal fractions obtained from Percoll density gradients. CDP-choline:Diacylglyceride-phosphorylcholine transferase (15) specific activity in Percoll isolated glyoxysomes was about 6% of that measured in ER preparations from sucrose gradients.

Cyt *c* oxidase activity in Percoll gradient isolated glyoxysomes

was typically less than 1% of that in mitochondria isolated on sucrose gradients. These levels of contamination were remarkably similar to the levels reported for glyoxysomes isolated on sucrose gradients (11). Antimycin A (10 μM) or cyanide (1 mM) affected rates of NADH:CCR in glyoxysomal preparations by less than 10% (data not shown), indicating that the mitochondrial contribution to electron transport activity was minimal in glyoxysomal fractions. NADP:6-P-gluconate dehydrogenase activity was not measurable in glyoxysomal preparations (data not shown). Thus, glyoxysomes isolated on Percoll gradients were only minimally contaminated with mitochondria, plastids, or ER.

Integrity of Isolated Glyoxysomes. MDH activity was stimulated 5- to 10-fold upon deliberate breakage of glyoxysomes by dilution with 0.225 M KCl for 30 min prior to assay. KCl added in isoosmotic medium had no effect on MDH activity (data not shown). The stimulation resulting from breakage is expressed as percent latency in Table II; MDH latencies of these preparations averaged 80%. The MDH activity measured in intact glyoxysomal suspensions was not sedimentable and was therefore extraglyoxysomal (data not shown). This indicates that the permeability of the glyoxysomal membrane to NADH and/or oxalacetate must limit the rate of MDH activity. These preparations of glyoxysomes were considered to be 80% intact and thus suitable for investigations of the orientation of the redox activities in the membrane.

Latencies of Redox Reactions. NADH:CCR and NADH:FCR were assayed in intact and deliberately broken glyoxysomes, and percent latencies were calculated as for MDH (Table II). Addition of Cyt *c* to intact glyoxysomes did not affect the integrity of the organelles as measured by MDH latency (data not shown). The effect of added ferricyanide on glyoxysomal integrity (MDH latency) could not be assessed due to interference by ferricyanide at 340 nm. When averaged over a number of independent preparations, with repetitive assays within preparations, the latencies of NADH:CCR and NADH:FCR were 30 to 40% (Table II). More significantly, reductase latencies averaged half of the latency measured for MDH in the same preparations.

Transhydrogenase Activity. We postulated that the glyoxysomal electron transport system may act as a transhydrogenase, oxidizing internal NADH with transmembrane electron transfer to cytosolic NAD or NADP. We tested this possibility using acetyl-analogs of NAD with deliberately broken organelles to eliminate limitations on enzyme rates imposed by impermeability of the glyoxysomal membrane (Table III). NADH oxidase activity in the absence of added electron acceptor in our preparations was about 10 nmol min^{-1} (mg protein)$^{-1}$. Rates of transhydrogenase activity were generally similar to the NADH oxidation rate measured in the absence of added electron acceptor (Table III). The rate of Cyt *c* reduction using acetyl-NADH was about half that using NADH (Table III).

Table II. *Comparison of Malate Dehydrogenase, NADH:FCR and NADH:CCR Activities in Intact and Deliberately Broken Glyoxysomes*

Numbers represent averages of means from three discrete glyoxysomal preparations, with three replications of each enzyme assay per preparation. Numbers in parentheses are standard errors of the means. Latency is defined as: 100 (broken rate − intact rate) (broken rate)$^{-1}$.

Enzyme	Specific Activity	Latency
	nmol·min^{-1}·mg^{-1} protein	%
MDH		80.3
Intact	5818 (3109)	
Broken	29557 (9427)	
NADH:CCR		37.1
Intact	22 (6)	
Broken	35 (12)	
NADH:FCR		44.7
Intact	406 (95)	
Broken	733 (216)	

Table III. *Rates of Electron Transfer between Pyridine Nucleotides and Their Acetyl-Analogs or Cyt c in Glyoxysomes*

Reactions were measured using disrupted glyoxysomes in column medium (see "Materials and Methods").

Donor	Acceptor				
	None	NAD	Acetyl-NAD	NADP	Cyt *c*
	nmol·min^{-1}·mg^{-1} protein				
NADH	10.9		10.9		189.6
Acetyl-NADH	2.7	2.1		2.2	98.0
NADPH			10.9		

Effectors of NADH:CCR Activity. NADH:CCR was assayed in glyoxysomes following incubation with various compounds reported to stimulate or inhibit electron transport activities in other membrane systems. Intact *versus* broken organelles were used to distinguish the effects with respect to the cytosolic *versus* matrix sides of the membrane.

Cibacron blue 3GA (Cibacron blue F-3GA; Procion blue; C.I. 61211), reported to inhibit NAD-requiring dehydrogenases (23), was a potent inhibitor of NADH:CCR in both intact and broken glyoxysomes at 50 μM (Table IV). NADH:FCR was inhibited to a similar degree by Cibacron blue 3GA (data not shown). Mersalyl inhibited the reductase activity in broken glyoxysomes by 50% at 50 μM, indicating sulfhydryl groups present on the enzyme.

Benzylaminopurine inhibited glyoxysomal electron transport to a lesser degree than has been documented for plant mitochondrial electron transport (3, 19). We tested the possible association of proton fluxes with glyoxysomal electron transport (16) with the ionophore FCCP. We observed no consistent effect of FCCP on NADH:CCR activity in intact glyoxysomal preparations (Table IV). However, 0.1% (w/v) BSA was present in these preparations to maintain glyoxysomal membrane integrity. BSA binds lipophilic compounds such as FCCP and thus may diminish their effectiveness.

Both 5′-ATP and 5′-ADP inhibited the reductase activity by 15 to 20% at a concentration of 1 mM. Neither 2′- nor 3′-mono- or diphosphorylated adenylates affected NADH:CCR (data not shown). Divalent cations, Mg^{2+} and Ca^{2+} (1 mM) stimulated NADH:CCR activity in intact glyoxysomes by 20%, while only the sulfate salts were stimulatory in broken organelles (Table IV). EGTA (equimolar) partially reversed the Ca^{2+}-induced stimulation (data not shown). K^+ salts had little effect at 1 mM on intact or broken organelles. The flavin antagonist quinacrine (atebrin) stimulated glyoxysomal electron transport at a much lower concentration than used in plasmalemma studies (5).

Trypsin Treatment of Intact Glyoxysomes. When intact glyoxysomes were incubated with trypsin in an isoosmotic medium, reductase activities were differentially affected. Trypsin treatment did not affect the MDH latency and hence the integrity of the organelles (Table V). Trypsin treatment did not decrease the activity of MDH measured in organelles which were deliberately disrupted following termination of proteolysis with trypsin inhibitor. This further demonstrated that the protease did not gain access to the interior of the organelles (Table V). NADH:CCR activity was essentially abolished by treatment of intact glyoxysomes with trypsin, while NADH:FCR was unaffected. In similar experiments, NADH:FCR was decreased slightly to trypsin treatment, to a degree approximately corresponding to the percentage of broken organelles estimated by MDH latency (data not shown). Attempts to reproduce these effects with a less specific protease (protease K) resulted in decreases in MDH latency, indicating loss of membrane integrity (data not shown).

Table IV. *Incubation of Glyoxysomes with Various Compounds: Effects on NADH:CCR Activity*

Glyoxysomes were preincubated for 30 min at 23°C in column buffer with indicated compound prior to assay.

Effector	Concentration	Intact	Broken
	μM	% control rate	
Cibacron blue	50	−80.9	−80.4
Quinacrine	250	+54.0	+41.7
Mersalyl	50	ND[a]	−50.0
Benzylaminopurine	500	ND	−16.7
FCCP	10	+5.0	ND
5′-ATP	1000	−15.4	ND
5′-ADP	1000	−20.6	ND
KCl	1000	−4.6	0.0
K_2SO_4	1000	ND	+7.5
$CaCl_2$	1000	+20.2	+4.5
$CaSO_4$	1000	+22.0	+22.0
$MgCl_2$	1000	+17.1	0.0
$MgSO_4$	1000	+17.5	+13.4

[a] Not determined.

Table V. *Glyoxysomal Integrity and Electron Transport Functions Following Trypsin Treatment*

Glyoxysomes (62.5 μg) were incubated in trypsin at a ratio of glyoxysomal protein:trypsin (w/w) 250:1, for 15 min, 23°C, in 0.5 M sucrose, 10 mM Hepes (pH 7.5), 0.1% dextran (incubation medium). The reaction was terminated with a 10-fold excess of soybean trypsin inhibitor, and enzyme assays conducted immediately in incubation medium. The enzyme activities shown are for broken organelles. Controls included trypsin but with trypsin inhibitor added prior to organelles.

Activity	Trypsin	Control
MDH latency[a]	81.3	80.0
MDH activity[b]	217.0	128.8
NADH:CCR[b]	0.00	0.38
NADH:FCR[b]	2.94	2.94

[a] % (see Table II). [b] nmol/min.

DISCUSSION

Glyoxysomes prepared by density gradient centrifugation in isoosmotic Percoll gradients and chromatography on Sepharose 2B were predominantly intact as estimated by MDH latency. The glyoxysomal preparations contained less than 1.0% contamination by mitochondria and were essentially free of plastids. More importantly, the glyoxysomes were only nominally contaminated with ER, demonstrating the glyoxysomal electron transport activities were greater than could be attributed to the 6% contamination by ER. The electron transport activities of Percoll gradient-isolated glyoxysomes were very similar to those measured in sucrose gradient-isolated glyoxysomes (11).

Redox reactions measured in whole glyoxysomes were generally insensitive to most electron transport inhibitors. The sensitivity of NADH:CCR to Cibacron blue 3GA indicated that the enzyme contains a pyridine-nucleotide binding site (23). NADH:CCR also contains (a) sulfhydryl group(s) sensitive to attack by the mercurial reagent mersalyl. Quinacrine has been reported as a flavin antagonist (5), but in our system consistently stimulated NADH:CCR. Bellion and Goodman (1) have reported that membrane potential-driven protein translocation in yeast peroxisomes is decreased by addition of a proton ionophore. We have detected an extraglyoxysomal increase in pH associated with NADH:CCR activity but not NADH:FCR (16). We speculated that redox-driven proton transport had occurred but observed little effect of FCCP on the pH change (DG Luster, RP Donaldson, unpublished data). FCCP also did not effect glyoxysomal NADH:CCR activity. The inhibition of reductase activity observed with adenylates was intriguing in light of the recent discovery of an ATPase in the yeast peroxisomal membrane (9).

The latencies observed for MDH, NADH:CCR, and NADH:FCR allow us to postulate the orientation of the electron donating (NADH) and accepting (Cyt *c*, ferricyanide) sites of the reductase enzymes in the membrane. The stimulation of redox activities observed upon dilution of glyoxysomes indicates that deliberate breakage of glyoxysomes permits access of impermeant substrates to redox enzyme catalytic sites within the intact organelle.

The substrates for these redox activities, NADH, ferricyanide, and Cyt *c*, have been considered to be impermeant to membranes (8). Cyt *c* is of a sufficient mass (12 kD), while ferricyanide is of sufficient mass and charge to render each impermeable. The high degree of MDH latency exhibited by intact glyoxysomes suggests that one or both of the substrates for MDH (NADH, oxalacetate) cannot penetrate the glyoxysomal membrane at a rate sufficient to maintain enzyme activity.

A greater amount of NADH:CCR and NADH:FCR activity was detected in preparations of organelles prior to deliberate disruption than could be accounted for by organelle damage, as estimated by comparison with MDH latency. If the glyoxysomal membrane is impermeable to NADH, then about half of the reductase activity would be occurring on the external surface of the intact organelle. Conversely, because the reductases do exhibit some latency, deliberate disruption seems to expose electron donor and/or acceptor sites facing the interior of the organelle.

An alternate explanation for the high MDH latency (substrates: NADH, oxalacetate) and lower reductase latencies (substrates: NADH, ferricyanide or Cyt *c*) is that NADH is permeant to some degree. This would imply that oxalacetate is of very limited permeability and thus responsible for MDH latency. Slow diffusion of externally presented NADH into the intact organelle *in vitro* would permit transmembrane reductase activities to proceed at rates decreased from those measured in broken organelles. The reductases could then transfer electrons to the impermeant external acceptors.

Information on pyridine nucleotide permeability in peroxisomes and glyoxysomes is limited, because the organelles are extremely fragile. NAD is apparently somewhat permeable to rat liver peroxisomes as measured by uptake studies followed by centrifugal filtration (24, 25). Studies with intact spinach leaf peroxisomes indicated permeability of the organelles to malate but a limited rate of NADH entry (27). Sucrose gradient-isolated glyoxysomes have been used for latency studies (8) and measurements of pyridine nucleotide content (6, 17). In all reports it has been concluded that glyoxysomes are of limited permeability to NADH.

It is important to emphasize that regardless of the assumption made concerning NADH permeability, the presence of both NADH:FCR and NADH:CCR activities measured in intact organelles demonstrates that the impermeant electron acceptors Cyt *c* and ferricyanide can accept electrons on the cytosolic face of the membrane. NADH donor sites could be on both sides or only the matrix side of the membrane.

The results from treatment of intact organelles with trypsin suggests that a trypsin sensitive domain or component of NADH:CCR faces the cytosol. A likely candidate for this component is Cyt b_5, which is present in the glyoxysomal membrane and is reduced by NADH (13). Cyt b_5 is a component of NADH:CCR activity in the rat liver ER (accepting electrons from a flavoprotein reductase) and is easily cleaved from the membrane by trypsin (22). Our experiments could not distinguish between the electron donor and acceptor site(s) of NADH:CCR, since proteolysis of either site would inactivate the

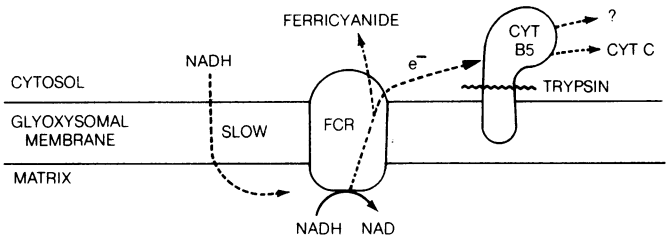

FIG. 2. Proposed orientation of electron transport components in the glyoxysomal membrane. e^-, electron.

enzyme. The latency of NADH:CCR together with sensitivity to externally presented trypsin suggest a transmembrane orientation for this redox activity (Fig. 2).

NADH:FCR activity was measurable in intact organelles, suggesting exposure of electron donor sites on the cytosolic face of the membrane. The site or component apparently lacks a trypsin-specific accessible segment. It is likely that NADH:FCR activity represents a flavoprotein dehydrogenase which transfer electrons to Cyt b_5 as in the ER membrane (10). Hicks and Donaldson (13) demonstrated that glyoxysomes contain flavin which is reducible by NADH.

We have used the electron acceptors Cyt *c* and ferricyanide to characterize the glyoxysomal redox system in an approach similar to that used in the investigations of ER electron transport (10). Endogenous electron acceptors for the glyoxysomal system have not been identified. Electrons generated by glyoxysomal metabolism may be transported to physiological acceptors such as chelated iron, ascorbate free radical, or Cyt b_5 in the outer mitochondrial or ER membranes.

We have presented in Figure 2 a model which represents the postulated orientation of glyoxysomal electron transport components based on the *in vitro* studies presented herein. The model assumes that the glyoxysomal membrane is somewhat permeable to NADH, and that Cyt *c* and ferricyanide are impermeant. We have omitted from Figure 2, but cannot exclude, the possibility that NADH is also oxidized on the cytosolic face of the glyoxysome.

Using the information from *in vitro* studies presented here, we postulate that glyoxysomal electron transport system may be coupled *in vivo* to β-oxidation and the glyoxylate cycle (11) with the export of electrons to external physiological acceptors. NADH generated by intraglyoxysomal metabolism could be oxidized on the matrix face of the membrane. The apparent K_m for NADH oxidation in whole glyoxysomes is on the order of 10 to 20 μM for the reductases (13). NADH would thus be oxidized before diffusing from the glyoxysome during β-oxidation, if electron acceptors are available. It does not appear likely that either transhydrogenase or NADH oxidase activity can sustain the rates of NADH turnover measured during β-oxidation (7). Thus, the physiological electron acceptor is apparently neither oxygen nor a cytosolic pool of oxidized pyridine nucleotides.

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