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# β-Oxidation and Glyoxylate Cycle Coupled to NADH: Cytochrome *c* and Ferricyanide Reductases in Glyoxysomes[1]

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## ABSTRACT

**Glyoxysomes isolated from castor bean (*Ricinus communis* L., var Hale) endosperm had NADH:ferricyanide reductase and NADH:cytochrome *c* reductase activities averaging 720 and 140 nanomole electrons/per minute per milligram glyoxysomal protein, respectively. These redox activities were greater than could be attributed to contamination of the glyoxysomal fractions in which 1.4% of the protein was mitochondrial and 5% endoplasmic reticulum. The NADH:ferricyanide reductase activity in the glyoxysomes was greater than the palmitoyl-coenzyme A (CoA) oxidation activity which generated NADH at a rate of 340 nanomole electrons per minute per milligram glyoxysomal protein. Palmitoyl-CoA oxidation could be coupled to ferricyanide or cytochrome *c* reduction. Complete oxidation of palmitoyl-CoA, yielding 14 nanomole electrons/per nanomole palmitoyl-CoA, was demonstrated with the acceptors, NAD, cytochrome *c*, and ferricyanide. Malate was also oxidized by glyoxysomes, if acetyl-CoA, ferricyanide, or cytochrome *c* was present. Glyoxysomal NADH:ferricyanide reductase activity has the capacity to support the combined rates of NADH generation by β-oxidation and the glyoxylate cycle.**

Triglycerides are converted to sucrose in fat storing tissues of germinating seeds such as the endosperm of castor bean. This conversion involves metabolism in glyoxysomes where the β-oxidation of fatty acids produces NADH plus acetyl-CoA (3). Acetyl-CoA is converted to succinate by the glyoxylate cycle, which also produces NADH. The NADH released during β-oxidation and the glyoxylate cycle in glyoxysomes does not have direct access to the mitochondrial electron transport system. Nevertheless, glyoxysomal NADH must be reoxidized so that a constant supply of NAD is available to accept electrons ($e^-$)[3] during β-oxidation and the glyoxylate cycle in the glyoxysome. The acyl-CoA oxidase of glyoxysomal β-oxidation avoids this by transferring $e^-$ directly to $O_2$ resulting $H_2O_2$.

Isolated glyoxysomal membranes contain NADH:CCR and NADH:FCR activities which can oxidize NADH (7, 9). The NADH generating dehydrogenases are matrix components of the glyoxysomes (7). Palmitoyl-CoA- and malate-dependent reductions of Cyt *c* and ferricyanide can be demonstrated when both glyoxysomal membranes and matrix are present *in vitro* (7). The hypothesized oxidation of NADH in glyoxysomes through the membrane reductases is an alternative to the proposed malate-aspartate shuttle (13). This shuttle would transport $e^-$ equivalents out of the glyoxysome in the form of malate, which would be oxidized in the mitochondria.

The purpose of this investigation was to determine if the glyoxysomal NADH:CCR and NADH:FCR are sufficiently active to support the oxidative activities of the glyoxysome. We examined the possibility that complete β-oxidation of palmitoyl-CoA might be linked to the reduction of either exogenous ferricyanide or Cyt *c*. We also measured glyoxysomal malate oxidation and its linkage to the membrane redox activities. Oxidation of NADH by the membrane $e^-$ transport system was compared to the malate-aspartate shuttle. Preliminary results have been published (5).

## MATERIALS AND METHODS

**Isolation of Glyoxysomes, Mitochondria, and ER.** Previously described techniques were used to isolate glyoxysomes and mitochondria from the endosperm of castor beans (*Ricinus communis* L., var Hale) germinated in darkness for 4.5 d at 30° C (4). Postnuclear supernatants were centrifuged on 34 to 56% (w/w) sucrose gradients for 25 min at 50,000*g* in a vertical rotor. The mitochondrial fractions were obtained at 40% sucrose. The most concentrated portions of the glyoxysomal fractions were collected from the 49 to 51% region of the gradients.

ER fractions were obtained at 30% sucrose after centrifugation of post-nuclear supernatants on gradients comprised of 2 ml 18%, 2 ml 20%, and a 20 ml linear gradient, 30 to 56% sucrose (9). All sucrose solutions were w/w and contained 1 mM EDTA (pH 7.5).

**Marker Enzymes.** Activities of the following enzymes were measured as previously described: catalase (8), isocitrate lyase (2), NADH:CCR and NADH:FCR (9), glutamate-OAA aminotransferase (2), Cyt *c* oxidase (14), phosphorylcholine-glyceride transferase (10). Protein was assayed by either of two methods (1, 11).

**Palmitoyl-CoA Oxidation.** β-Oxidation was measured as palmitoyl-CoA dependent NADH generation. The 1 ml reaction contained 30 mM phosphate buffer (pH 7.5), 0.15 mg BSA, 0.01% Triton X-100, 0.1 mM CoA, 0.2 mM NAD, 0.02 to 0.2 mg of glyoxysomal protein. The reaction was initiated by adding 1 to 10 nmol palmitoyl-CoA and NADH formation was followed at 340 nm using an extinction coefficient of 6.22 $mM^{-1}$. The concentration of the palmitoyl-CoA stock solution was determined at 259 nm using an extinction coefficient of 16 $mM^{-1}$.

Palmitoyl-CoA dependent Cyt *c* or ferricyanide reduction was measured by including in the above reaction mixture either 0.1 mM Cyt *c* or 1 mM $K_3Fe(CN)_6$. The concentrations of Cyt *c* and ferricyanide were higher than usual to allow palmitoyl-CoA oxidation to go to completion. The reduction of Cyt *c* was determined at 550 nm using an extinction coefficient of 20 $mM^{-1}$.

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[3] Abbreviations: $e^-$, electron; NADH:CCR, NADH:Cyt *c* reductase; NADH:FCR, NADH:ferricyanide reductase; OAA, oxalacetate; BSA, fatty acid-free bovine serum albumin.

The reduction of ferricyanide was determined at 420 nm using an extinction coefficient of 1 $mM^{-1}$.

**Malate Oxidation.** Malate oxidation was measured in a 1 ml reaction mixture containing 10 mM K-phosphate (pH 7.4), 0.5 mM $MgCl_2$, 0.2 mM NAD, 0.1 mM acetyl-CoA, and 0.02 to 0.2 mg of glyoxysomal protein. NADH formation was determined at 340 nm. Malate dependent Cyt *c* reduction was measured at 550 nm in the above mixture with the addition of 0.04 mM Cyt *c*. Malate dependent ferricyanide reduction was measured at 420 nm in a mixture where the Cyt *c* was replaced by 1 mM $K_3Fe(CN)_6$. The reactions were initiated by adding 1 mM malate.

**Oxygen Uptake by Glyoxysomes Plus Mitochondria.** Glyoxysomes and mitochondria were pelleted from the postnuclear supernatant by centrifugation at 10,000*g* for 10 min. The pellet was resuspended in 0.4 M sucrose, 5 mM $MgSO_4$, 10 mM K-phosphate (pH 7.2), 0.1% (w/v) BSA (reaction medium). Oxygen uptake was measured using a Clark oxygen electrode at 30° C in a 2 ml reaction medium containing 0.05 mg glyoxysomal protein with the addition of substrate or substrate plus effector. The substrate concentration used was 1.5 mM NADH, 12.5 mM succinate, or 0.1 mM palmitoyl-CoA, and the effector was 0.15 mM ADP, 0.1 mM KCN, or 0.1 mM aminooxyacetic acid.

## RESULTS AND DISCUSSION

**Redox Activities in Isolated Glyoxysomes Compared to Contamination by ER or Mitochondria.** Percentages of ER and mitochondrial marker enzyme activities in the glyoxysomal fractions were used to determine the levels of cross-contamination. The Cyt *c* oxidase activity, relative to protein, in the glyoxysomes was 1.4% of that in the mitochondria and glyoxysomal phosphorylcholine glyceride transferase was 5% of the ER activity (Table I). Isocitrate lyase and catalase activities in the mitochondrial and ER fractions were less than 5% of the glyoxysomal levels. The glyoxysomal activity of NADH:FCR was 25% of the ER value and 24% of the mitochondrial value. That is, glyoxysomal NADH:FCR was 5 times greater than would be expected from ER contamination. NADH:CCR in the glyoxysomes was 15% of that in the ER and 96% of the antimycin A insensitive activity in the mitochondria. The glyoxysomal reductase activities relative to protein were expected to be less than the ER activities because 90 to 95% of the glyoxysomal protein is matrix rather than membrane (7).

Table I. *Redox Activities in Glyoxysomes Compared to Marker Enzymes for Mitochondria and ER*

Mitochondrial reductase assays included 0.04 mM antimycin A and 0.1 mM KCN. These had no significant effect on the activities in the glyoxysomes and ER (data not shown). Units are nmol/min except for phosphorylcholine glyceride transferase, pmol/min.

| | Glyoxysomes | Mitochondria | ER |
|---|---|---|---|
| | *units/mg protein*[a] | | |
| Catalase | 2189 ± 296 | 15 ± 8 | 17 ± 17 |
| | $n = 4$[b] | $n = 5$ | $n = 5$ |
| Isocitrate lyase | 607 ± 48 | 4 ± 3 | 33 ± 25 |
| | $n = 5$ | $n = 5$ | $n = 5$ |
| NADH:FCR | 722 ± 269 | 3020 ± 497 | 2927 ± 906 |
| | $n = 5$ | $n = 4$ | $n = 4$ |
| NADH:CCR | 143 ± 52 | 165 ± 66 | 971 ± 321 |
| | $n = 8$ | $n = 7$ | $n = 5$ |
| Cyt *c* oxidase | 72 ± 38 | 5241 ± 1093 | 38 ± 10 |
| | $n = 4$ | $n = 3$ | $n = 5$ |
| Phosphorylcholine glyceride transferase | 26 ± 9 | 20 ± 5 | 525 ± 156 |
| | $n = 4$ | $n = 4$ | $n = 6$ |

[a] Values are means ± SD. [b] Number of experiments.

**Glyoxysomal Redox Activity Coupled to Oxidations of Palmitoyl-CoA and Malate.** The rate of NADH:FCR in isolated whole glyoxysomes was greater than the combined rates of palmitoyl-CoA and malate oxidation using NAD as the acceptor (Table II). The malate oxidation activity with NAD in glyoxysomes was sufficient to synchronize $\beta$-oxidation with the glyoxylate cycle which incorporates two molecules of acetyl-CoA per cycle (Fig. 1). The rate of palmitoyl-CoA oxidation coupled to ferricyanide reduction was greater than when NAD was the only acceptor. NADH:CCR activity was less than NADH:FCR or $\beta$-oxidation and was probably limited by the amount of glyoxysomal Cyt $b_5$ needed to transfer $e^-$ to Cyt *c* (9).

**Complete Fatty Acid Oxidation in Glyoxysomes.** Palmitoyl-CoA oxidation went to completion, yielding 14 $e^-$ per molecule of palmitoyl-CoA as expected (Table III). When malate was added, 16 additional $e^-$ were obtained from palmitoyl-CoA. This indicated that 8 molecules of acetyl-CoA were released and condensed with OAA to form citrate, allowing the oxidation of malate to OAA (Fig. 1). During the process malate dehydrogenase generated 8 NADH or 16 $e^-$. These data indicate that malate oxidation can be linked to fatty acid oxidation within glyoxysomes. CoA and NAD were required for palmitoyl-CoA oxidation. Glyoxylate partially replaced CoA because the malate synthase reaction releases free CoA (Table III). Oxidations of other substrates also approached completion: 6.0 nmol NADH per nmol oleoyl-CoA and 2.6 nmol NADH per nmol octanoyl-CoA (not shown).

Table II. *Glyoxysomal $\beta$-Oxidation and Glyoxylate Cycle Coupled to Reductions of Cyt c and Ferricyanide*

| Donors | Acceptors | | |
|---|---|---|---|
| | NAD | Cyt *c* | Ferricyanide |
| | *nmol $e^-$/min·mg protein*[a] | | |
| NADH | ND[b] | 143 ± 52 | 722 ± 269 |
| | | $n = 8$ | $n = 5$ |
| Palmitoyl-CoA | 337 ± 49 | 48 ± 14 | 560 ± 94 |
| | $n = 6$[c] | $n = 4$ | $n = 3$ |
| Malate | 226 ± 40 | 76 ± 8 | 87 ± 24 |
| | $n = 4$ | $n = 7$ | $n = 4$ |

[a] Values are presented as means ± SD. [b] Not determined. [c] Number of experiments.

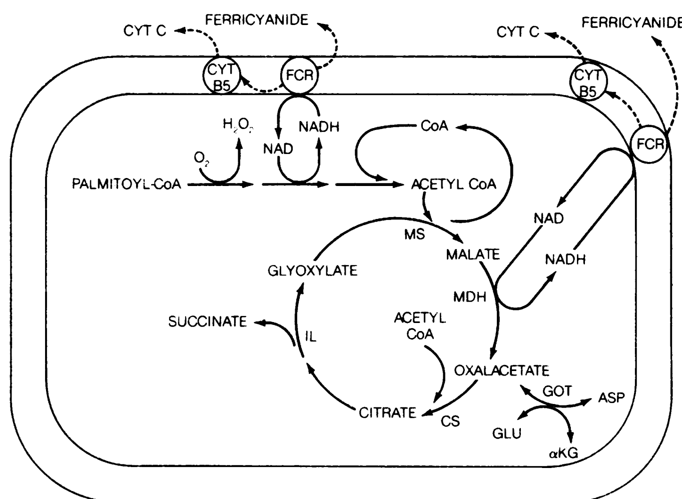


FIG. 1. Proposed linkage of $\beta$-oxidation and malate oxidation to membrane redox enzymes in glyoxysomes. Malate synthase (MS), malate dehydrogenase (MDH), ferricyanide reductase (FCR), citrate synthase (CS), glutamate-OAA aminotransferase (GOT), isocitrate lyase (IL).

Table III. *Extent of Palmitoyl-CoA Oxidation in Glyoxysomes*

A reference cuvette containing half the concentration of Cyt *c* or ferricyanide was used to allow measurement of the high *A* values. The reaction was followed to completion, 15 min for NAD, 40 min for ferricyanide, and 134 min for Cyt *c*. Malate (1 mM) or glyoxylate (1.5 mM) was added where indicated.

| | Acceptor | | |
|---|---|---|---|
| | NAD | Cyt *c* | Ferricyanide |
| | *nmol e⁻/nmol Palm-CoA* | | |
| Complete | 14.5 | 15.0 | 15.1 |
| Add Malate | 30.1 | ND[a] | ND |
| Omit CoA | 0.7 | 2.7 | 1.4 |
| Omit CoA, add Glyoxylate | 10.6 | 9.5 | ND |
| Omit NAD | 1.8 | 0.0 | 1.8 |
| Omit Glyoxysomes | 0.1 | 0.6 | 2.2 |
| Omit Palmitoyl-CoA | 0.0 | 3.6 | 2.2 |

[a] Not determined.

Table IV. *Malate Oxidation in Glyoxysomes*

Acetyl-CoA was omitted except where indicated. The concentrations of the additions were 0.1 mM acetyl-CoA, 1 mM glutamate, 2 mM $\alpha$-ketoglutarate ($\alpha$-KG), 2 mM aspartate (Asp), 5 mM OAA, and 0.2 mM aminooxyacetic acid (AOA). The omission of malate, NAD, Cyt *c*, ferricyanide, or glyoxysomes resulted in rates of less than 6 nmol $e^-$/min mg protein.

| Condition | Acceptor | | |
|---|---|---|---|
| | NAD | Cyt *c* | Ferricyanide |
| | *nmol e⁻/min mg protein* | | |
| Control | 2.0 | 30.0 | 123.0 |
| Acetyl-CoA | 226.1 | 73.7 | 121.0 |
| Glutamate | 244.6 | 69.0 | 337.0 |
| $\alpha$-Ketoglutarate | ND[a] | 38.0 | 162.0 |
| Aspartate | ND | 38.0 | 148.0 |
| OAA | ND | 5.0 | 5.0 |
| $\alpha$-KG + Asp | ND | 0.0 | 6.0 |
| $\alpha$-KG + Asp + AOA | ND | 32.0 | 96.0 |

[a] Not determined.

Palmitoyl-CoA oxidation linked to Cyt *c* or ferricyanide reduction also went to completion and required CoA. NAD was also needed, indicating that the endogenous NAD (4) did not provide an optimal concentration in the reaction mixture. Had acyl-CoA oxidase donated $e^-$ to ferricyanide or Cyt *c* the yield would have been 28 nmol $e^-$ per nmol palmitoyl-CoA; this was not observed.

**Malate Oxidation Coupled to Transamination and Redox Activities in Glyoxysomes.** The malate dehydrogenase reaction alone does not favor malate oxidation (Table IV). However, NAD dependent malate oxidation was observed when acetyl-CoA was available to condense with OAA, form citrate, and thus relieve product inhibition (Fig. 1). Alternatively, the addition of glutamate provided for the conversion of the OAA to aspartate via the glutamate-OAA aminotransferase (2). Oxidation of malate also occurred if either Cyt *c* or ferricyanide was added. This indicated that the NADH generated by the malate dehydrogenase was oxidized by the NADH:CCR or the NADH:FCR. The coupling of malate oxidation to the redox activities was enhanced by the addition of acetyl-CoA or glutamate and inhibited by OAA (Table IV). Cyt *c* or ferricyanide dependent malate oxidation was also inhibited by the presence of $\alpha$-ketoglutarate and aspartate but restored by the aminotransferase inhibitor, aminooxyacetate. The activity of the glutamate-OAA aminotransferase was found to be 247 nmol/min·mg glyoxysomal protein and was inhibited 89% by 0.2 mM aminooxyacetate (not shown). This aminotransferase may buffer a low OAA concentration within glyoxysomes to promote malate oxidation.

**Oxygen Uptake by Glyoxysomes plus Mitochondria.** The malate-aspartate shuttle proposed by Mettler and Beevers (13) may be responsible for oxidizing some glyoxysomal NADH *in vivo*. This would involve transport of malate from the glyoxysomes to mitochondria, oxidation of malate to OAA, and transamination to aspartate, which would return to the glyoxysomes. Aspartate would combine with $\alpha$-ketoglutarate to yield OAA and glutamate.

We investigated the relationship between glyoxysomal $\beta$-oxidation and mitochondrial oxygen uptake using a resuspended pellet containing both organelles. The rate of NADH oxidation was 10 nmol O/min and with the addition of ADP was 19 nmol O/min. Succinate oxidation was 128 nmol O/min and was completely inhibited by KCN. Palmitoyl-CoA dependent net oxygen uptake, 27 nmol O/min, was not inhibited by cyanide. This would indicate that mitochondrial oxygen uptake was blocked while net glyoxysomal oxygen consumption was doubled by inhibition of catalase (2, 3). The aminotransferase inhibitor, aminooxyacetate, also had no effect on palmitoyl-CoA dependent oxygen consumption by glyoxysomes plus mitochondria. This indicated that the malate-aspartate shuttle was not functioning under these *in vitro* conditions.

The malate-aspartate shuttle depends upon glyoxysomal aminotransferase activity which we found to be about 250 nmol/min·mg glyoxysomal protein. This is just sufficient to match the total rate of oxidation required by $\beta$-oxidation plus the glyoxylate cycle, about 500 nmol $e^-$/min·mg glyoxysomal protein. This shuttle mechanism would require regulated transport of malate, aspartate, glutamate, and $\alpha$-ketoglutarate through the glyoxysomal and mitochondrial membranes at appropriate rates. Such movements have yet to be demonstrated. Malate transport and oxidation could be rate limiting as it is in the metabolism of spinach leaf peroxisomes (18).

It seems unlikely that glyoxysomal NADH oxidation would be accomplished by the rapid transport of NADH through the glyoxysomal membrane. Exogenous NADH is not readily oxidized by intact glyoxysomes indicating that it does not pass through the membrane at an appropriate rate (6, 12). Also, isolated glyoxysomes retain NAD and NADP (4). Intact spinach leaf peroxisomes are permeable to malate at a rate that will support hydroxypyruvate reductase but the rate of NADH permeation is not sufficient (18). Liver peroxisomes take up NAD *in vitro*, but the rate of transport has not been compared to the rate of $\beta$-oxidation (16). Glyoxysomes and peroxisomes may be slowly permeable to NAD to provide for changes in concentration of the co-factor pool within the organelles. The NADH:CCR and NADH:FCR in the glyoxysomal membrane could prevent the escape of NADH because $K_m$ for NADH is very low, 20 $\mu$M (9).

If the glyoxysomal membrane reductases are coupled to $\beta$-oxidation and the glyoxylate cycle *in vivo*, then extraglyoxysomal acceptors must be available to receive the $e^-$ from NADH. Ferricyanide is not a natural acceptor and Cyt *c* is located within the mitochondria. A cytosolic acceptor, for example ascorbate, could mediate the transport of electrons between the glyoxysomes and the outer mitochondrial membrane as in the chromaffin granule system (17). Cyt $b_5$ in the outer mitochondrial membranes or ER may serve as the acceptor for the glyoxysomal $e^-$. Antimycin insensitive NADH:CCR activity, indicating the presence of Cyt $b_5$, has been detected in the outer membranes of mitochondria from castor bean endosperm (15).

*In vivo* electron transport through the glyoxysomal membrane would require an appropriate orientation of the NADH:FCR in the membrane. Only half of the NADH:FCR is measurable in

demonstrably intact organelles, indicating that electron donor and/or acceptor sites are on both sides of the membrane (12). The oxidation of NADH in glyoxysomes by the membrane redox system may allow β-oxidation and the glyoxylate cycle to be partially uncoupled from mitochondrial oxidative phosphorylation. NADH oxidation by the malate-aspartate shuttle would link β-oxidation to higher levels of ATP generation. Thus, the balance of electron flow through the two routes would depend on the demand for ATP as fatty acid is oxidized and converted to sucrose in castor bean endosperm.

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