Preferential oxidation of glycine by the respiratory chain of pea leaf mitochondria

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1. INTRODUCTION

The oxidation of glycine by mitochondrial glycine decarboxylase (EC 2.1.2.1) represents an important step in the photorespiratory cycle of leaf tissue. NADH generated in this reaction must be re-oxidised in order for the cycle to continue. Re-oxidation may occur either via the mitochondrial respiratory chain [1,2] or via a reversal of malate dehydrogenase in the presence of OAA [2,3]. The latter system could supply necessary reducing power to the peroxisomes during photorespiration, if linked to an OAA/malate shuttle across the mitochondrial and peroxisomal membranes.

In view of increasing evidence for the continued operation of the tricarboxylic acid cycle in the light [4], we investigated the potential competitive effects of tricarboxylic acid cycle substrates on the oxidation of glycine by pea leaf mitochondria, with the aim of determining which system of re-oxidation of glycine NADH is more likely to occur in vivo.

2. MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* cv. Massey Gem) were grown in trays of vermiculite in a glasshouse for 10–13 days. All biochemicals were purchased from Sigma Chemicals (St Louis MO).

Pea leaf mitochondria were isolated according to [5]. Protein was estimated as in [6] with BSA as standard and chl as in [7]. Mitochondrial protein was corrected for the contribution by broken thylakoids by assuming a thylakoid:protein ratio of 6.9:1, for these preparations [8].

Oxygen consumption was measured polarographically in 2 ml standard reaction medium (0.3 M sorbitol, 10 mM TES buffer, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 0.1% BSA, pH 7.2) using a Rank O_2 electrode at 25°C.

NH_3 release was measured simultaneously with O_2 consumption in a sealed perspex vessel into which were fitted an Orion ammonia electrode (connected to a Beckman pH meter) and a Clark-type O_2 electrode (Yellow Springs OH). The signals from both electrodes were monitored with a twin-channel Rikadenki recorder. The capacity of the vessel was 9 ml. Mitochondria (1.5–2.0 mg protein) were added to 8.5 ml standard reaction medium (see above). Other additions were made as indicated in table legends. NH_3 release was calibrated by injecting known amounts of NH_4Cl.

External NADH oxidation was measured at 340 nm in a 2 mm light path cuvette containing a 0.6 ml aliquot removed from a Rank O_2 electrode containing 2.5 ml standard reaction medium, mitochondria (0.6 mg protein), 0.19 mM NADH,

Abbreviations: BSA, bovine serum albumin; chl, chlorophyll; TPP, thiamine pyrophosphate chloride; Mal, malate; Glu, glutamate; 2-OG, 2-oxoglutarate; OAA, oxaloacetate

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9 mM glucose and 2 enzyme units of hexokinase. The reaction mixture was incubated for 3 min prior to the initiation of rapid NADH oxidation by the addition of 0.3 mM ADP.

3. RESULTS

Mitochondria isolated from pea leaves demonstrated good respiratory control [9] and ADP:O ratios of 2.0:2.2 for glycine and 2.2:2.5 for malate. Cyanide-insensitive oxygen uptake did not exceed 15% of the total oxidative activity of these mitochondria.

The close coupling of glycine oxidation and the respiratory chain in pea leaf mitochondria is well illustrated in table 1. State 3–state 4 transitions in O_2 uptake and NH_3 release, could be demonstrated on the addition of limited amounts of ADP, with ratios of O_2 consumed to NH_3 released being of the order of 1:2, as predicted from theoretical considerations [1].

The effects of various respiratory substrates on glycine oxidation in state 3 are shown in table 2. The 'predicted' rate of O_2 uptake represents the summation of O_2 uptake rates measured with the individual substrates. The use of an ADP-generating system (glucose + hexokinase) allowed for the maintenance of the state 3 condition over extended periods of time (e.g., 15–20 min) thus ensuring that respiratory substrates attained their maximum rates of oxidation following their addition to the medium. Glycine oxidation in state 3 was unaffected by the presence of other respiratory substrates which could be expected to compete at either the NAD (e.g., malate, 2-oxoglutarate) or respiratory chain level (e.g., succinate, NADH). Oxygen uptake, however, was markedly stimulated, demonstrating that concurrent oxidation of substrates did occur. The discrepancy between the predicted rate and the observed rate of O_2 uptake, indicates the extent of inhibition of oxidation of the other respiratory substrates in the presence of glycine (in no case was NH_3 release from glycine inhibited). The rate of malate oxidation, for example, was inhibited 30% in the presence of glycine. This inhibition was confirmed by directly measuring the decarboxylation of [^{14}C]malate in the presence and absence of glycine (not shown, see [10]). Similarly, external NADH oxidation was inhibited about 27% in the presence of glycine (table 3), although concurrent oxidation of succinate, malate or 2-oxoglutarate (not shown) also inhibited external NADH oxidation. The

Table 1

Simultaneous measurement of O_2 uptake and NH_3 release during the oxidation of glycine by pea leaf mitochondria

	NH_3 release (nmol.mg protein^{-1}.min^{-1})	O_2 uptake (nmol.mg protein^{-1}.min^{-1})
Glycine + ADP (state 3)	156	81
State 4	99	49
+ ADP	163	78
State 4	92	46

Assays contained 11 mM glycine and 0.17 mM ADP

Table 2

Effect of various respiratory substrates on glycine oxidation by pea leaf mitochondria in state 3

	NH_3 release	O_2 uptake	Predicted O_2 uptake
	(nmol.mg protein^{-1}.min^{-1}		
Glycine	150	71	
+ Mal, Glu, TPP	150	142	171
Glycine	120	64	
+ Succinate, NADH	120	192	277
Glycine	100	51	
+ 2-OG, TPP, malonate	100	118	120
Glycine	125	63	
+ Mal, Glu, TPP, succinate, NADH	130	247	376

Assays contained 11 mM glycine, 11 mM glucose, and 4 units of hexokinase. State 3 was initiated by the addition of 0.28 mM ADP. Following the establishment of a steady state rate, additions were made to the following final concentrations: 11 mM malate, 11 mM glutamate, 11 mM succinate, 11 mM 2-oxoglutarate, 0.8 mM NADH, 0.1 mM TPP and 5 mM malonate. Oxidation rates for succinate and NADH were 83 and 130 nmol.mg protein^{-1}.min^{-1}, respectively

Table 3

Effect of glycine, succinate and malate on external NADH oxidation

	O_2 uptake			NADH oxidation (½)	
	Measured	Predicted	Deficit	Measured	Control-measured
	(nmol.mg protein^{-1}.min^{-1})				
Exp.1					
NADH	212	–	–	216	0
NADH + glycine	252	308	56	158	58
NADH + succinate	242	335	93	158	58
NADH + succinate, glycine	267	427	160	97	119
Exp.2					
NADH	175	–	–	175	0
NADH + Mal, Glu, TPP	201	249	48	132	43
NADH + Mal, Glu, TPP, glycine	225	326	101	96	79

Substrates were incubated with NADH for 3 min prior to initiation of state 3 with ADP. All substrates were present at 11 mM except TPP (0.1 mM) and malonate (5 mM). Phthalonate (5 mM) was present in assays containing malate to inhibit external malate dehydrogenase-mediated NADH oxidation resulting from the export of OAA during malate oxidation. Note the rate of NADH oxidation is halved to make it equivalent to O_2 consumption

presence of $CaCl_2$ which sometimes activates NADH oxidation by pea leaf mitochondria [11] had no effect on the relative degree of inhibition of external NADH oxidation caused by these substrates. In the presence of multiple substrates, not all of the difference between the predicted and observed rates of O_2 uptake could be accounted for by decreases in NADH oxidation alone; thus the oxidation of other substrates was also inhibited. Using this method, succinate oxidation was found to be inhibited in the presence of NADH, while malate oxidation was only markedly restricted in the presence of both glycine and NADH (table 3).

There is some uncertainty as to whether mitochondrial electron transport is shut down in the light or not [12], but even if electron transport does operate in the light, it is unlikely that cellular respiration would be operating under state 3 conditions [13]. Rather, it is probable that in vivo respiratory rates are somewhere between the state 3 and state 4 rates [14]. We therefore examined the effect of some respiratory substrates on glycine oxidation in state 4. Even under these restricted respiratory conditions, glycine is preferentially ox-

Table 4

Effect of malate and 2-oxoglutarate on glycine oxidation by pea leaf mitochondria in state 4

	NH_3 release	O_2 uptake	Predicted O_2 uptake
	(nmol.mg protein^{-1}.min^{-1})		
Glycine + ADP	138	68	–
State 4	86	43	
Mal, Glu, TPP + ADP	–	102	
State 4	–	47	
+ Glycine	86	76	90
2-OG, TPP, malonate + ADP	–	71	
State 4	–	32	
+ Glycine	88	65	75

Assays contained 11 mM glycine, 11 mM malate, 11 mM 2-oxoglutarate, 0.1 mM TPP, 5 mM malonate and 0.17 mM ADP

Table 5

Accessibility of various NADH-generating mitochondrial dehydrogenases to OAA-dependent re-oxidation by malate dehydrogenase

Substrate	Assumed enzyme	O_2 uptake (nmol.mg protein^{-1}.min^{-1})	
		State 3	+ OAA
Glycine	Glycine decarboxylase	114	10
2-OG, TPP, malonate	2-Oxoglutarate dehydrogenase	45	12
Citrate	Isocitrate dehydrogenase	28	6
Glutamate	Glutamate dehydrogenase	27	0
Mal, arsenite	Malic enzyme[a]	40	10

[a] Possibly some malate dehydrogenase also

Assays contained 11 mM glycine, 2-oxoglutarate, citrate, glutamate and malate, 5 mM malonate and 2 mM arsenite. State 3 was initiated by the addition of 0.7 mM ADP followed by the addition of 0.7 mM OAA

idised by the electron transport chain at the expense of the other respiratory substrates present (table 4). State 4 malate oxidation (as in state 3, table 2) was inhibited 30% upon the addition of glycine. The glycine state 4 rate, however, as judged by NH_3 release, was unaffected by the presence of other respiratory substrates.

It is well established that the addition of OAA to leaf mitochondria oxidising glycine, leads to a dramatic inhibition of O_2 uptake while maintaining the rate of NH_3 release at a similar or even increased rate [3,5,15]. This is attributed to the rapid re-oxidation of NADH by malate dehydrogenase and re-cycling of the NAD to glycine decarboxylase. If such a system were to operate in vivo, one might expect there to be a specialised degree of interaction between the two enzyme systems involved (i.e., glycine decarboxylase and malate dehydrogenase) to ensure the efficient removal of reducing equivalents in this way. However, this does not appear to be the case as the results in table 5 indicate that OAA-dependent re-oxidation of NADH occurs with a range of mitochondrial substrates, demonstrating that malate dehydrogenase has equal access to intramitochondrial NADH regardless of which enzyme generates the NADH. That is, there is no specific compartmentation of malate dehydrogenase within the matrix of pea leaf mitochondria.

4. DISCUSSION

This evidence shows that pea leaf mitochondria preferentially oxidise glycine when confronted with a mixture of glycine and tricarboxylic acid cycle substrates. Even under state 4 (ADP-limited) conditions, when competition among substrates for electron transport could be expected to be most severe, glycine oxidation was not diminished (table 4). Therefore, it appears that the electron-transport chain of pea leaf mitochondria has an absolute preference for the NADH generated from glycine oxidation. This preference also extends to the competition between external NADH and glycine (table 3). In contrast, glycine oxidation by rat-liver mitochondria was severely restricted in the presence of respiratory substrates such as malate, succinate and 2-oxoglutarate [16]. The mechanism by which glycine is preferentially oxidised via the respiratory chain is yet to be elucidated, but may be related to the structural association of glycine decarboxylase with the inner mitochondrial membrane and thus the respiratory chain.

Another mechanism for re-oxidising intramitochondrial NADH is to reduce OAA via malate dehydrogenase. It has been suggested that NADH generated during glycine oxidation suffers this fate in order to transfer the reducing equivalents to peroxisomes during photorespiration. A number

of substrate shuttles have been postulated [3,9,15]. Oxaloacetate does cause oxidation of intramitochondrial NADH, but does so in a general manner, without any apparent preference for a particular substrate.

Irrespective of the actual mechanism involved, it is essential that glycine oxidation be maintained for the continued operation of the photorespiratory cycle. It might thus be expected that the in vivo mechanism would show a preference for glycine. Our results show that the respiratory chain but not malate dehydrogenase, has a preference for NADH generated from glycine. Therefore, we suggest that in vivo, the oxidative decarboxylation of glycine is linked to the mitochondrial electron-transport chain rather than a metabolic shuttle involving OAA and malate dehydrogenase. This proposal further implies that the mitochondrial electron-transport chain is operating in the light.

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