THE INTRACELLULAR LOCALIZATION OF THE GLYCOLLATE-OXIDISING ENZYME OF *ANABAENA CYLINDRICA*

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1. Introduction

Glycollate-oxidising enzymes have been reported from several blue-green algae, namely *Anabaena flos-aquae* and an *Oscillatoria* species [1,2], *Anabaena cylindrica* [3,4] and *Anacystis nidulans* [5]. Their role in the metabolism of exogenous- [3,6] and photosynthetically-produced glycollate [7,8] has received attention, although little is known about the intracellular location of blue-green algal glycollate-oxidising enzymes.

Grodzinski and Colman [1] reported that most of the enzyme activity in extracts of *Anabaena flos-aquae* and *Oscillatoria* sp. was located in a clear-blue 20 000 *g* supernatant fraction, although a little activity remained in the residual green pellet. We have determined the intracellular distribution of the *Anabaena cylindrica* glycollate-oxidising enzyme and present evidence that it is largely associated with the thylakoids. The significance of our findings is discussed in terms of comparative glycollate oxidation in plants and of the locale of respiratory electron transport in the prokaryotic photosynthetic cell.

2. Materials and methods

2.1. *Organism and growth conditions*

Anabaena cylindrica Lemm. (Fogg's strain) from the Culture Collection of Algae and Protozoa, Cambridge, was grown axenically in the medium of Allen and Arnon [9] supplemented with 1 mM NH_4Cl. Culture conditions were as detailed previously [3].

2.2. *Preparation of cell extracts*

4 l of exponential-phase cells were harvested by centrifugation at 500 *g* for 20 min and washed in 0.75 M tricine HCl buffer pH 7.5, containing 0.01 M NaCl. After recentrifugation and resuspension in this buffer to about 45 ml, the cells were broken using a French pressure cell [10] at 16 000 lb per in^2. The broken cell suspension was immediately diluted with and equal volume of tricine buffer containing 0.4 M sucrose. Cell disruption and all subsequent centrifugation procedures were performed at 0–4°C.

2.3. *Fractionation of cell extracts*

Unbroken cells were removed by bench centrifugation at 2500 *g* for 15 min. The pellet was discarded and the supernatant used. Aliquots of this and all subsequent fractions were retained for assay, the sediments being gently resuspended in tricine buffer containing 0.4 M sucrose.

2.4. *Differential centrifugation*

The 2500 *g* cell-free supernatant was re-spun at 2500 *g* for 30 min to yield a pellet of cell wall fragments. The resulting supernatant was spun in an MSE 18 centrifuge at 35 000 *g* for 30 min to sediment the thylakoid membranes. The pellet was gently resuspended and washed twice by repeated spins at 35 000 *g* for 30 min. All thylakoid washings were retained for assays. The first 35 000 *g* supernatant was spun at 100 000 *g* for 1 hr in an MSE Superspeed 50 to produce sediment and soluble fractions.

2.5. *Density gradient centrifugation*

2 ml aliquots of the twice-washed 35 000 *g* thylak-

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oid fraction were layered onto 20 ml linear gradients of 10 to 60% (w/w) sucrose in tricine buffer in polycarbonate tubes. Centrifugation was performed in the MSE Superspeed 50 at 80 000 *g* for 4 hr. 1 ml fractions were collected from the bottom of the tubes after puncture with an MSE tube piercer.

2.6. *Assay for glycollate oxidation*

Glycollate oxidation was measured by following oxygen consumption in a Rank Pt-Ag oxygen electrode as described previously [4]. Respiratory electron transport chain inhibitors were added as detailed in the Results and discussion section.

Absorbance at 663 nm as a measure of chlorophyll a was determined on a Pye Unicam SP 800 B spectrophotometer. Protein was determined by the Lowry procedure [11] using bovine serum albumin as a standard and interference by sucrose in protein assays of density gradient fractions, accounted for [12].

2-Heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) was obtained from Sigma, St Louis, Missouri, USA. All other chemicals were from British Drug Houses, Poole, Dorset, England, and were used at the highest purity commercially available.

3. Results and discussion

As in our earlier investigations [4] *Anabaena cylindrica* extracts catalyzed glycollate-dependent oxygen uptake, producing glyoxylate. The specific activities of the differential centrifugation fractions are shown in table 1. Enzyme activity was found in all fractions except the 35 000 *g* supernatant and 100 000 *g* fractions derived from it. The highest specific activities were exhibited by the 35 000 *g* pellet and its derivatives. Washing of this sediment caused the release of enzyme but resulted in successively higher specific activities of the 35 000 *g* pellet. On a percentage basis, the 35 000 *g* pellet retained the highest proportion of the enzyme originally present in the 2500 *g* cell-free supernatant (table 1).

The 35 000 *g* pellet showing glycollate oxidase activity contains chlorophyll *a*, catalyzes Photosystem II and Photosystem I reactions (measured as the ferricyanide-Hill reaction and ascorbate/2,6-dichlorophenolindophenol Mehler reaction using methyl viologen, respectively) and contains thylakoid membranes [13]. The results in table 1 thus suggest that high glycollate oxidase is associated with the thylakoid membranes of *A. cylindrica*. Indeed, the distribution of enzymic glycollate oxidation coincided with that of particulate chlorophyll *a*, measured as absorbance at 663 nm, in centrifuged linear sucrose density gradients of the thylakoid fraction (fig.1).

Table 1
Differential centrifugation of *Anabaena cylindrica* extracts[a]

Fraction		Specific Activity[b]	% of total activity
2500 *g* × 15 min pellet		0.000	0
2500 *g* × 15 min supernatant		0.255	100
2500 *g* × 30 min pellet		0.770	8
2500 *g* × 30 min supernatant		0.358	82
35 000 *g* × 30 min pellet		2.316	50
35 000 *g* × 30 min supernatant		0.000	0
100 000 *g* × 1 hr pellet		0.000	0
100 000 *g* × 1 hr supernatant		0.000	0
35 000 *g* × 30 min washings of original 35 000 *g* × 30 min pellet:			
First	pellet	3.618	48
	supernatant	2.921	9
Second	pellet	5.728	48
	supernatant	5.618	11

[a] Extracts were prepared by disruption of cells in 0.075 M tricine HCl buffer pH 7.5 containing 0.01 M NaCl using a French pressure cell at 16 000 lb/in^2 followed by dilution with an equal volume of buffer containing 0.4 M sucrose. Enzyme activity was measured as glycollate-dependent oxygen uptake, according to Codd and Stewart [4].

[b] Specific activty is μmol O_2 consumed/hr mg protein^{-1}.

The location of oxidative electron transport in the prokaryotic photosynthetic cell is an intriguing concept. Cytochemical studies of the blue-green algae *Nostoc sphaericum* and an unnamed *Anabaena* sp. by Bisalputra et al. [14] indicated that the thylakoids were the site of both photosynthetic and respiratory electron transport. Oelze and Drews [15] have more recently reviewed the evidence that a common intracytoplasmic membrane system is involved in both types of electron transport in members of the *Rhodospirillaceae*. Our findings of the association of glycollate oxidation, coupled to oxygen consumption,

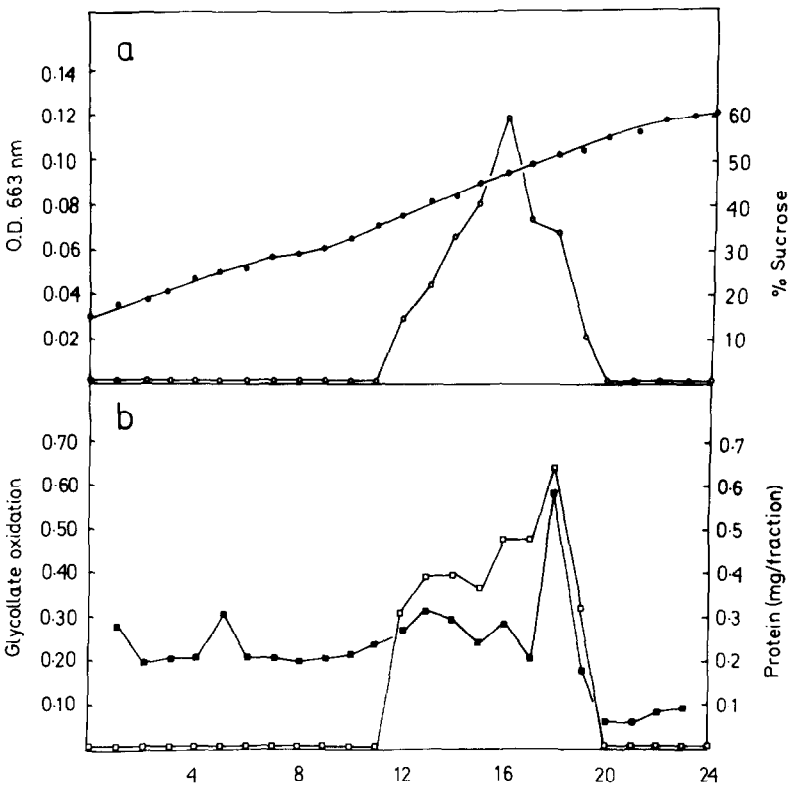

Fig.1. Distribution of *Anabaena cylindrica* chlorophyll *a* and glycollate oxidase activity after sucrose density gradient centrifugation of the twice-washed 35 000 *g* × 30 min pellet. 20 ml gradients of sucrose in tricine buffer were loaded with 2 ml of the washed thylakoid fraction which initially showed a specific activity of 5.728 μmol oxygen consumed/hr mg protein^{-1} (see table 1). Centrifugation and fractionation was performed as in Materials and methods and the fractions are numbered in the reverse order in which they were collected. (a) (○–○) Chlorophyll *a* distribution, measured as optical density at 663 nm (●–●) Sucrose concentration (% w/w). (b) (□–□) Glycollate oxidation (μmol oxygen consumed/hr mg protein^{-1}. (■–■) Protein concentration.

with a membrane system raises the possibility that membrane-bound electron carriers may be involved in glycollate oxidation leading to oxygen consumption in *A. cylindrica*.

We have therefore determined the effects of several inhibitors of respiratory electron transport on glycollate oxidation by the thylakoid membranes. As shown in table 2, HOQNO at 1 μg/ml final concentration caused complete inhibition. Potassium cyanide at 2×10^{-4} M caused a 40% inhibition of glycollate-dependent oxygen uptake and at 1 mM, caused complete inhibition. Similar results were obtained with sodium azide (table 2).

The rudimentary understanding of the respiratory electron transport system in blue-green algae [16,17] and in *A. cylindrica* in particular, renders speculative

Table 2
The effect of inhibitors on *Anabaena cylindrica* glycollate oxidase[a]

Inhibitor	Concentration	% Inhibition[b]
2-Heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO)	1 μg/ml	100
Potassium cyanide	$2 \cdot 10^{-4}$ M	40
	$1 \cdot 10^{-3}$ M	100
Sodium azide	$2 \cdot 10^{-4}$ M	37
	$1 \cdot 10^{-3}$ M	100

[a] Inhibitors were added directly to the oxygen electrode assay to give the final concentration shown.

[b] All assays were run using the 35 000 *g* × 30 min (thylakoid) pellet material and contained 0.74 mg protein. The specific activity of the enzyme was 2.12 μmol oxygen consumed/hr mg protein^{-1}.

the interpretation of the effects of these inhibitors. However, if the respiratory electron transport system of *A. cylindrica* involves *b*-type cytochromes, then the inhibition by HOQNO would suggest a site of entry of electrons from glycollate at the level of cytochrome *b* [18]. The inhibitory effects of potassium cyanide and sodium azide, both well-established as inhibitors of cytochrome *c* oxidases in blue-green algae [16,17], clearly show that glycollate oxidation associated with the thylakoid membranes can link to oxygen via the terminal electron carriers of the respiratory electron transport system.

Previous experiments have established that the glycollate-oxidising enzyme of *A. cylindrica* can function with an artificial electron acceptor (2,6-dichlorophenolidophenol) and that other unknown cellular oxidant(s) can also serve, since glycollate oxidation can occur under circumstances where oxygen uptake is not measureable [4]. However, when assayed as oxygen consumption, most of the enzyme activity is located in the thylakoid membrane fraction (table 1) and this glycollate oxidation remains firmly associated with the thylakoids during density gradient centrifugation (fig.1). Since this particulate glycollate oxidation is linked probably via respiratory electron carriers to oxygen, the enzyme may be termed a glycollate oxidase. Analogies may be drawn between our findings with the prokaryotic *A. cylindrica* and the glycollate-oxidising enzyme of *Escherichia coli,* which can couple indirectly to oxygen probably involving cytochromes [19]. Further comparisons may be drawn between this concept of a prokaryotic respiratory glycollate oxidase, associated in the case of *A. cylindrica* with the thylakoid membranes, with the glycollate-oxidising enzyme of several eukaryotic algae. Stabenau has reported [20] that glycollate oxidation in homogenates of the alga *Chlorogonium elongatum* is mainly found in the mitochondrial fraction, and on the basis of inhibitor studies suggests that glycollate oxidation in vivo may couple to oxygen via the mitochondrial electron transport system. Similarly, a diatom enzyme, which when coupled to oxygen is cyanide-sensitive [21], is localized in the mitochondria and operationally involves cytochromes [22]. The enzyme present in *A. cylindrica, E. coli* and the eukaryotic algal mitochondria thus contrasts with the cyanide-insensitive [23] peroxisomal glycollate oxidase of higher plants and some algae [24].

Finally, this communication provides evidence that the thylakoid membranes of *A. cylindrica* are a site of oxidative electron transport. Whether some distinct areas of the membranes are associated with photosynthetic electron transport and others with respiratory electron transport, as may occur in the *Rhodospirillaceae* [15], or whether regions and even electron carriers are shared by the two processes in *A. cylindrica* remains to be determined.

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