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Subcellular Distribution of Enzymes of Glycolate Metabolism in the Alga *Cyanidium caldarium*[1]

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ABSTRACT

The intracellular distribution of enzymes capable of catalyzing the reactions from phosphoglycolate to glycerate in the bluegreen colored eucaryotic alga *Cyanidium caldarium* has been studied. After separating the organelles from a crude homogenate on a linear flotation gradient, the enzymes glycolate oxidase and glutamate-glyoxylate aminotransferase along with catalase were present in the peroxisomal fraction (density: 1.23 grams per cubic centimeter). Serine hydroxymethyltransferase was found in the mitochondrial fraction (density: 1.18 grams per cubic centimeter). In contrast to the observations in green leaves of higher plants, the enzymes for the conversion of serine to glycerate (serine-glyoxylate aminotransferase and hydroxypyruvate reductase) were found only in the soluble fraction of the gradient. The partial characterization of enzymes from *Cyanidium* participating in glycolate metabolism revealed only slight differences from the corresponding enzymes from higher plants. The phylogenetic implications of the observed similarities between the enigmatic alga *Cyanidium* and higher plants are discussed.

The alga *Cyanidium caldarium* (Tilden) Geitler is unique among eucaryotic organisms because of its ability to grow in very acid media (below pH 1), at high temperatures (up to 56°C), and supplied with pure CO_2 only. The taxonomic position of *Cyanidium* is still unresolved. Many scientists include *Cyanidium* in the red algae, but there are also reports that it could be a bridge alga between the Cyanophytes and the Rhodophytes (25), or even a cyanelle-possessing alga (18). Ultrastructural studies have shown that *Cyanidium* possesses a nucleus, one chloroplast, and one mitochondrion. Dictyosomes and vacuoles are absent. In contrast to that, *Cyanidium* contains several microbodies (21). Since nothing is known about the role of these microbodies in the metabolism in *Cyanidium*, it was of interest to study the function of these organelles.

MATERIALS AND METHODS

Algal Material and Growth Conditions

Cyanidium caldarium strain 107.79 was obtained from the Algensammlung Göttingen (FRG). If not stated otherwise, cultures were grown autotrophically in glass tubes at 35°C in continuous light (80 $\mu E/m^2/s$) and supplied with air only. The nutrient medium was the same as that of Ford (11). Cells were harvested at late log phase by low speed centrifugation.

Organelle Separation

All steps were carried out at about 4°C. For organelle separation cells, approximately 4 g wet weight, were washed consecutively in 8 and 16% (w/w) sucrose in TT-buffer. (TT-buffer: 25 mM Tris-TES buffer [pH 7.8], 1 mM EDTA, 1 mM $MgCl_2$, 1 mM KCl). Then the cells were transferred to 20 mL of TT-buffer containing 16% (w/w) sucrose, 6 mM DTT, 0.2% (w/v) BSA, and 1% (w/v) PVPP[3]). After adding 20 mL glass beads (0.2 mm), cells were broken in a Virtis homogenizer (30–120 s). The resulting homogenate was filtered through nylon-mesh (50 μm) and centrifuged at 1,500*g* for 15 min in a swinging bucket rotor. Powdered sucrose was then added slowly to the supernatant to give a final concentration of 57% (w/w). This homogenate (about 15 mL) was poured into a centrifuge tube and a 57 to 30% linear sucrose gradient was layered on top. Gradient solutions contained 1 mM EDTA and 1 mM DTT (pH 7.5). After centrifuging in a Beckman SW 27 rotor at 65,000*g* for 15 h, the gradient was collected in 1.2 mL fractions.

Preparation of Cell Extracts

Cell extracts were prepared by homogenizing about 5 g (wet weight) material in 4 to 5 volumes of chilled TT-buffer, containing 1% (w/v) PVPP together with 25 mL glass beads in a Virtis homogenizer. The resulting homogenate was spun at 30,000*g* for 30 min and the supernatant used for enzyme assays. For phosphoglycolate phosphatase and serine hydroxymethyltransferase a grinding medium described by James and Schwartzbach (16) was used instead of TT-buffer.

Partial Purification of Hydroxypyruvate Reductase

The method used for partial purification of hydroxypyruvate reductase was essentially that of Husic and Tolbert (15). After ammonium sulfate precipitation (45–70% saturation), the sample was desalted and loaded onto an Affi-Gel blue column (Bio-Rad). The HPR was eluted from the column with a linear KCl gradient, the peak fractions were pooled

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[3] Abbreviations: PVPP, polyvinylpolypyrrolidone; Ches, (2-[*N*-cyclohexylamino]-ethanesulfonic acid; HPR, hydroxypyruvate reductase.

and concentrated. The concentrated enzyme fraction was then loaded onto a Sephacryl S-300 column. HPR fractions from the S-300 column were concentrated and loaded onto a Sephadex G-200 column. The peak fractions from this column were pooled, concentrated, and used for subsequent characterization.

Determination of Native Mol Wt

The native mol wt of the hydroxypyruvate reductase was estimated by gel filtration on a Sephacryl S-300 column (1 × 80 cm) using catalase (240,000), fumarase (194,000), glucose oxidase (186,000), aldolase (161,000), alcohol dehydrogenase (141,000), α-amylase (50,000), peroxidase (40,000), and Cyt *c* (12,500) as markers. (All proteins were purchased from Sigma.)

Assays

All assays for measuring enzyme activities were carried out at 25°C. Enzymes were tested as described: catalase, fumarase, malate dehydrogenase (26), citrate synthase (14) after removal of DTT by ultrafiltration (Centricon-10, Amicon), glycolate oxidase (formation of phenylhydrazone as well as of H_2O_2), glutamate-glyoxylate aminotransferase, enoyl-CoA hydratase (13), serine hydroxymethyltransferase (32), aldolase, alcohol dehydrogenase, α-amylase, peroxidase (2), hydroxypyruvate reductase, pyruvate reductase (15), phosphoglycolate phosphatase (16). The assay mixture for serine-ketoacid aminotransferase contained 25 mM Ches-KOH (pH 9.0), 0.1 mM pyridoxal-5-phosphate, 2 mM Li-hydroxypyruvate, enzyme, and 2 mM amino acid. After 0, 15, and 30 min the reaction was stopped by adding 0.75% (w/v) TCA. Protein was removed by centrifugation and the amino acids were extracted from the supernatant using Sephadex SP-25 (23). Serine produced was converted to glyoxylate using periodate and then was determined enzymically using lactic dehydrogenase (Sigma) (2). In the reverse reaction the formation of [1-^{14}C] glycine from [1-^{14}C]glyoxylate (Amersham) was determined as described by Walton (30). For the immunotitration of glycolate oxidase, 40 μL of increasing concentrations of antiserum against glycolate oxidase from spinach leaves (a generous gift from Dr. C. R. Somerville) and 50 μL glycolate oxidase from spinach leaves or *Cyanidium* were incubated at 0°C for 20 min. Then, 5 μL of a 10% suspension of protein A-*Staphylococcus aureus* cells were added. After 5 min the cells were spun down (5 min at 3000*g*) and the supernatant assayed for glycolate oxidase activity. Protein was determined by the Coomassie blue method (5) using BSA as standard. Chl content was determined by the method of Böger (4). (Unless stated otherwise, chemicals were purchased from Sigma.)

RESULTS

In the alga *Cyanidium*, seven enzymes participating in glycolate metabolism could be demonstrated: phosphoglycolate phosphatase, glycolate oxidase, catalase, glutamate-glyoxylate aminotransferase, serine hydroxymethyltransferase, serine-glyoxylate aminotransferase, and hydroxypyruvate reductase (Table I).

It is frequently emphasized that in algae the typical glycolate oxidizing enzyme is a dehydrogenase rather than an oxidase as in higher plants. This is certainly true for the Chlorophytes, Bacillariophytes, and Euglenophytes (12), but recent studies have shown that in Charophytes (27), Xanthophytes (13), and *Cyanophora* (3) a glycolate oxidase is present. The finding that the enigmatic alga *Cyanidium* also possesses a glycolate oxidase suggests that among the algae a glycolate oxidase is more widespread than previously assumed. The properties of five of the glycolate pathway enzymes from *Cyanidium* exhibited many similarities to the corresponding enzymes from higher plants, as shown below.

Phosphoglycolate Phosphatase

The phosphoglycolate phosphatase from *Cyanidium* showed a broad pH optimum between pH 5.5 and 7.5 using Tris-Mes buffer. The K_m for phosphoglycolate was 93 μM using an enzyme fraction obtained after ammonium sulfate fractionation (50–80% saturation). In crude extracts the K_m was found to be much higher (about 6 mM). No phosphatase activity was detected in the crude extract using p-nitrophenylphosphate as substrate, indicating that the high K_m in crude extracts was not due to nonspecific phosphatases.

Glycolate Oxidase

The properties of the glycolate oxidase from *Cyanidium* are summarized in Table II. The results show that the enzyme is similar to the higher plant enzyme (9, 12). Figure 1 shows that about 1.2 μL of spinach-glycolate oxidase antiserum was needed to precipitate half of the glycolate oxidase from spinach. The addition of 40 μL of the same antiserum to glycolate oxidase from *Cyanidium* had no effect on the activity.

Glutamate-glyoxylate Aminotransferase

The pH optimum for the aminotransferase was 8.0 to 8.5 using Hepes buffer. The K_m for glyoxylate was about 0.5 mM in crude extracts at pH 8.0.

Serine-pyruvate Aminotransferase

In crude extracts of *Cyanidium*, 12 amino acids were found that can serve as amino donor for the formation of serine from hydroxypyruvate (Table III). The pH optimum for serine formation was between 8.0 and 9.5. Because the transferase capable of converting hydroxypyruvate to serine was not purified, it is not clear whether one or more aminotransferases are present in *Cyanidium*. Of all amino donors tested, the lowest K_m and the highest velocity were obtained for alanine. Glycine, asparagine, and glutamate can serve as amino donor in decreasing order of preference. Therefore, the measured activity could be due to a serine-pyruvate aminotransferase as found in parsley leaves (31), spinach leaves (19), and the alga *Mougeotia* (32). In gradient fractions, however, where a more sensitive assay was necessary (see below), the aminotransferase was tested in the reverse reaction using glyoxylate

Table I. *Effect of Growth in High CO_2 on the Activity of Enzymes of the Glycolate Pathway*

Cultures were grown at 45°C for 7 d supplied with air and 100% CO_2, respectively. Enzyme activity expressed in nmol/min/mg protein.

Growth Conditions	Air	100% CO_2	Ratio
Fresh weight	6.05 g/L	12.54 g/L	1:2.1
Extracted protein	93 mg	220 mg	1:2.3
Phosphoglycolate phosphatase	372	193	1:0.52
Catalase	262×10^3	71×10^3	1:0.37
Glycolate oxidase	60.4	27.0	1:0.45
Glu-glyoxylate aminotransferase	85.2	46.5	1:0.55
Serine hydroxymethyltransferase	75.3	34.6	1:0.46
Ser-glyoxylate aminotransferase	48.1	28.7	1:0.60
HPR[a]	504	312	1:0.62
Citrate synthase	268	267	1:1
Fumarase	115	243	1:2.1
Malate dehydrogenase	540	918	1:1.7
Enoyl-CoA hydratase	365	367	1:1
Pyruvate reductase	162	765	1:4.7

[a] HPR was tested at suboptimal substrate concentration (0.1 mM), to avoid interference by pyruvate reductase, which also can reduce hydroxypyruvate but possesses a lower substrate affinity (data not shown).

Table II. *Some Properties of the Glycolate Oxidase from Cyanidium*[a]

Property	Value
pH optimum	8.0–8.5
K_m for glycolate	0.2 mM
Production of H_2O_2	Positive[b]
Inhibition by 2 mM KCN	Negative
Substrate specificity[b]	Velocity (%)[c]
Glycolate	100
Glyoxylate	17
L-lactate	15
D-lactate	2

[a] The enzyme was partially purified by ammonium sulfate fractionation. [b] Activity assayed using the aminoantipyrine-peroxidase test. [c] At 5 mM substrate concentration; 100% = 252 nmol/min/mg protein.

as acceptor. Thus under these conditions the enzyme is described as a serine-glyoxylate aminotransferase.

Hydroxypyruvate Reductase

The HPR from *Cyanidium* was purified about 60-fold (Table IV). Some properties of the purified enzyme are shown in Table V. For comparision properties of the corresponding enzyme from higher plants are also listed.

It can be seen that the properties of the HPR from *Cyanidium* are similar to the higher plant enzyme with regard to the mol wt, pH optimum, and substrate affinity. However, differences were noticed in the reaction velocity in the presence of NADH and NADPH. Whereas the enzyme from spinach consistently shows a higher or equal velocity with NADH than with NADPH over the whole pH range, the activity of the HPR from *Cyanidium* was more than two times higher at low pH in the presence of NADPH (Fig. 2).

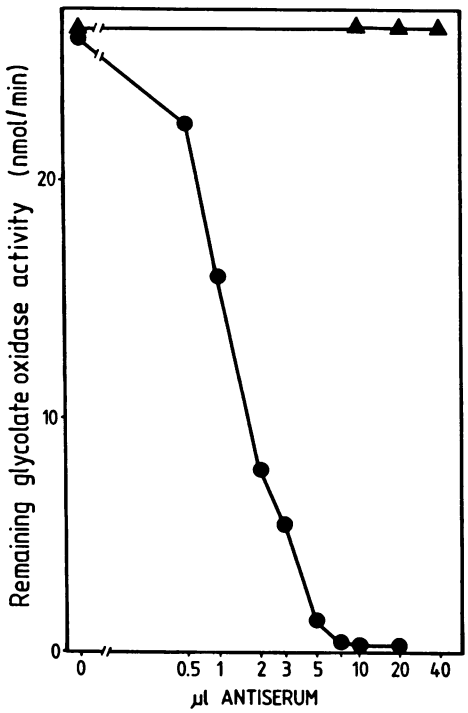


Figure 1. Immunotitration of glycolate oxidase from *Cyanidium* (▲) and spinach (●) with antiserum against glycolate oxidase from spinach.

A nonperoxisomal NADPH-HPR recently isolated by Klecskowski and Randall (17) from spinach leaves exhibits a similar pH optimum, but the substrate/cosubstrate specificity as well as the pH optimum with NADH are quite different from the *Cyanidium* enzyme. The high activity with NADPH found in *Cyanidium* was not due to a contaminating glyoxylate reductase as indicated by the low activity with glyoxylate. No activity was detected when pyruvate was used as substrate.

Table III. *Serine-ketoacid Aminotransferase: Effectiveness of Different Amino Acids in the Hydroxypyruvate Transaminase Reaction*

The pH used was 9.0 for *Cyanidium* and spinach and 8.0 for *Mougeotia* and parsley. Concentration (mM) of amino donor was 2 for *Cyanidium* and spinach, 20 for *Mougeotia*, and 17 for parsley. Concentration (mM) of OH-pyruvate was 2 for *Cyanidium*, 12 for Mougeotia, and 17 for parsley.

Amino Donor	K_m	Velocity			
		Cyanidium	Spinach[a]	*Mougeotia*[b]	Parsley[c]
	mM	%			
Alanine	2.2	100[d]	100	100	100
Glycine	3.0	73	61	18	47
Asparagine	4.7	73	51	23	
Glutamate	7.4	61	23	8	1
Histidine		22	<10	7	
Aspartate		18	16	9	0
Lysine		13	<10	7	
Glutamine		12	20	12	
Valine		8	<10	9	
Phenylalanine		7	<10	7	
Leucine		7	<10	7	0
β-Alanine		6			0
Methionine		<5	<10	2	
Proline		<5	<10	4	
Arginine		<5	<10	5	

[a] Data from Liang *et al.* (19). [b] Data from Winkler *et al.* (32). [c] Data from Willis and Sallach (31). [d] 100% = 67 nmol/min·mg^{-1} protein.

The ratio of the activity with NADH and NADPH did not change after purification of the enzyme. Addition of NADH together with NADPH to the reaction mixture gave about 88% of the activity found with NADH alone. Column chromatography and native PAGE also gave no indication that the HPR from *Cyanidium* exists in two forms with different nucleotide requirements (data not shown).

Separation of Organelles

A successful separation of the organelles from *Cyanidium* could only be obtained by letting the organelles float to their density during prolonged centrifugation in a linear sucrose gradient; all other centrifugation techniques led to high trapping effects. Figure 3 shows that the mitochondria, chloroplast fragments, and microbodies could be separated as indicated by the distribution of citrate synthase/fumarase, Chl, and catalase, respectively. In addition to catalase, the microbodies contain glycolate oxidase and glutamate-glyoxylate aminotransferase (Fig. 3c). Glycine produced by the action of these two enzymes can be converted to serine by serine hydroxymethyltransferase in the mitochondria (Fig. 3b). No hydroxypyruvate reductase or serine-glyoxylate aminotransferase could be demonstrated within the gradient. These enzymes were found only in the soluble fraction, *i.e.* at the bottom of the flotation gradient (Fig. 3d).

It should be noted that the activity of the serine-ketoacid aminotransferase was less than 5% of that of HPR. A similar ratio was reported for spinach by Liang *et al.* (19).

No malate dehydrogenase activity was found that corresponded to the catalase peak at 1.23 g/cm^3 (data not shown).

Activity of Glycolate Pathway Enzymes in Cultures Grown in Air and in High CO_2

When cells were grown in high CO_2 and thus presumably with lower rates of photorespiration, the specific activity of the HPR and six other enzymes of glycolate metabolism decreased by 38 to 63% (Table I). The similar response of all seven enzymes to high CO_2 conditions is seen as an indication that these enzymes participate in the same pathway. The activity of five enzymes not linked to the photorespiratory pathway did not decrease in cells grown in high CO_2.

DISCUSSION

The glycolate pathway (2 glycolate → glycerate + CO_2) in higher plants is well established and seems to be basically identical in C_3, C_4, CAM, and aquatic plants. In algae this pathway exhibits a number of variations. In green algae two different glycolate pathways were found: Charophytes seem to possess a pathway very similar to that of higher plants (27). Other green algae were found to convert glycolate to glycerate exclusively in the mitochondria (27), where the glycolate oxidizing enzyme is a dehydrogenase rather then an oxidase as in the Charophytes. Bacillariophytes (22), Cyanobacteria (8), and Euglenophytes (20) also possess a glycolate dehydrogenase, but other enzymes participating in glycolate metabolism show differences among these groups. In the Xanthophyte *Bumilleriopsis* another variation of this pathway was observed. This alga possesses a glycolate oxidase, but the second key enzyme, hydroxypyruvate reductase, is absent (13).

Therefore, it was surprising to find that the bluegreen-colored alga *Cyanidium* contains all the enzymes known from higher plants to be necessary for the conversion of phosphoglycolate to glycerate. The key enzyme glycolate oxidase

Table IV. *Purification of the Hydroxypyruvate Reductase*

Fraction	Volume	Enzyme Activity	Specific Activity	Protein	Yield	Purification
	mL	*units*[a]	*units/mg*	*mg*	%	*-fold*
Supernatant 1 h/30,000*g*	50.0	196	0.65	302	100	1
Ammonium sulfate 45–70% saturation	16.8	112	2.73	41	57	4
Affi-Gel blue pool	2.0	88.4	11.6	7.6	45	18
S-300 pool	1.75	76.0	38.0	2.0	39	58
G-200 pool	1.0	37.1	39.0	0.95	19	60

[a] μmol/min.

Table V. *Some Properties of the Hydroxypyruvate Reductase from Cyanidium, and Comparison with the Enzyme from Higher Plants*

Property	*Cyanidium*	Higher Plants[a]
Native mol wt	~90,000	91–97,000
pH Optimum		
In the presence of NADH	6.5	6.0–7.0
In the presence of NADPH	3.5–4.5	5.1
Substrate inhibition	>2 mM	>1 mM; >2 mM
K_m for hydroxypyruvate		
In the presence of 0.2 mM NADH	56 μM at pH 6.5 107 μM in crude extracts	50–62 μM 117–222 μM
In the presence of 0.2 mM NADPH	3000 μM at pH 6.5 1200 μM at pH 5.0 700 μM at pH 4.0	2630 μM
K_m for glyoxylate		
In the presence of 0.2 mM NADPH	6.15 mM at pH 6.5	5.7–15.5 mM
K_m for the cosubstrate (at pH 6.5, [hydroxypyruvate] = 1.8 mM)		
In the presence of NADH	39 μM	3–12 μM
In the presence of NADPH	211 μM	
Velocity[b]		
at pH 6.5		
In the presence of NADH	100%	100%[c]
In the presence of NADPH	28%	12%
at pH 5.0		
In the presence of NADH	64%	64%
In the presence of NADPH	110%	54%
at pH 4.5		
In the presence of NADH	57%	24%
In the presence of NADPH	121%	24%

[a] Data from Tolbert *et al.* (29), Gerhardt (12), Titus *et al.* (28), Anderson and Emes (1). [b] At 1 mM hydroxypyruvate concentration; 100% = 38 μmol/min/mg protein. [c] Data calculated from a pH curve published by Tolbert *et al.* (29).

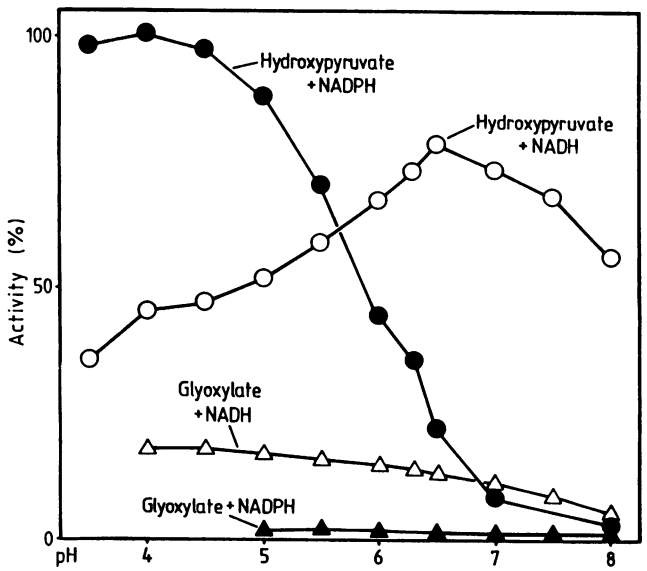
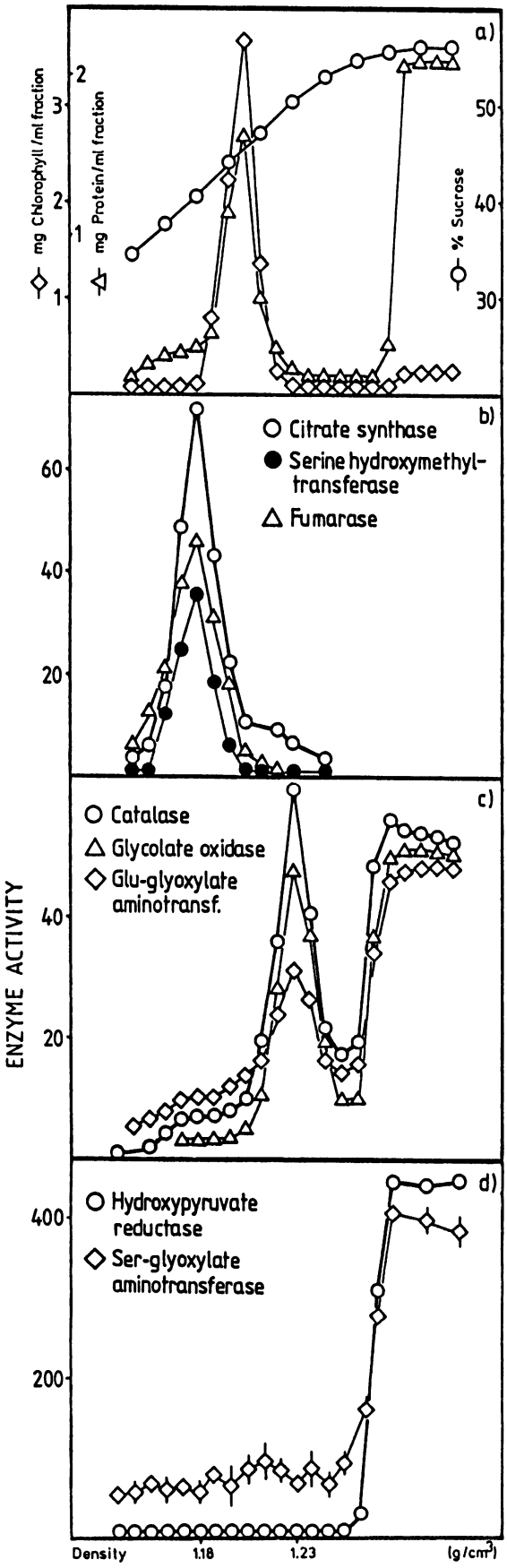


Figure 2. Effect of pH on the activity of hydroxypyruvate reductase with NADH or NADPH as cosubstrate. (Buffers used: pH 8.0–5.5; 0.1 M potassium phosphate; pH 5.5–3.5; 50 mM acetic acid/KOH plus 0.1 M KH_2PO_4. Substrate concentrate = 1.5 mM; cosubstrate concentrate = 0.2 mM; 100% = 36 μmol/min/mg protein).

showed kinetic properties that are similar to the enzyme from higher plants. The enzyme HPR, however, was found to possess some properties not shared by the enzyme from spinach. In *Cyanidium* this enzyme could be found only in the soluble fraction of the sucrose gradient. Because several attempts to isolate intact chloroplasts from *Cyanidium* failed, it cannot completely be ruled out that the enzyme originates from chloroplasts (or other organelles) broken during the homogenization. Whereas, the mol wt, the substrate affinity, and the pH optimum of the HPR showed close resemblance to the enzyme from higher plants, the velocity of the enzyme in the presence of NADPH was quite high. Although the HPR from *Cyanidium* exhibited the highest activity at acid pH with NADPH as cosubstrate, the K_m for hydroxypyruvate under these conditions is more than 10 times higher than with NADH at neutral pH. It was reported by Enami *et al.* (10) that the intracellular pH in *Cyanidium* remained in the range from 6.8 to 7.0 even when the external pH was shifted from 1.2 to 8.4. These data support the assumption that under physiological conditions NADH is the likely reductant for the HPR.

In crude extracts of *Cyanidium* hydroxypyruvate aminotransferase activity was found with a number of amino acids. The order of preference for the amino donor was similar to



that of serine-pyruvate aminotransferases from higher plants. However, it seems likely that the *Cyanidium* extract contains more than one aminotransferase since in cells of higher plants it is known that several aminotransferases with low substrate specificities are present (12). No attempt was made to purify the aminotransferase(s) from *Cyanidium*. No aminotransferase was found within the gradient using a more sensitive assay in which the formation of ^{14}C-glycine from ^{14}C-glyoxylate with serine as donor was measured. We refer to this activity as serine-glyoxylate aminotransferase, although we recognize that this activity could also be brought about by a serine-pyruvate aminotransferase (19, 31, 32).

Thus, both the activity of serine-glyoxylate aminotransferase and HPR were found exclusively in the soluble fraction of the gradient. Therefore we assume that in *Cyanidium* the conversion of serine to glycerate occurs in the cytosol. This stands in contrast to the situation in higher plants where these reactions always occur in the peroxisomes.

Although some distinct differences exist, the similarities between the glycolate pathway enzymes from higher plants and from *Cyanidium* are striking. Unfortunately, this finding does not help to solve the taxonomic position of *Cyanidium*. To assume that *Cyanidium* might be closely related to the ancestors of higher plants can be excluded, because this alga possesses several ultrastructural and biochemical features that are unknown from higher plants or green algae. However, assuming that *Cyanidium* is an endocyanome, the host cell could well be a colorless green alga, because the most notable differences between this group and *Cyanidium* are the chloroplast structure and the pigmentation (24). The concept of *Cyanidium* as a cyanelle-possessing alga was suggested by several authors (6, 18), mainly to help explain the unique features of this alga. Other authors strongly rejected this concept and felt that there is sufficient evidence to assign *Cyanidium* to the Rhodophytes (7, 24, 25). Although the endocyanome concept seems to lack support from ultrastructural and biochemical studies, it is interesting that—as in *Cyanidium*—a glycolate oxidase was also found in the alga *Cyanophora* (3), the typical example for an endocyanome.

The most likely explanation for our findings, however, would be the polyphyletic origin of the glycolate pathway enzymes. The occurrence of a glycolate oxidase in Charophycean and Xanthophycean algae as well as in *Cyanophora* and *Cyanidium*—algal groups that seem to share almost no phylogenetic significant markers—suggests a polyphyletic origin of this enzyme. The lack of cross-reactivity of the *Cyanidium* enzyme with antibodies against the glycolate oxidase from spinach and the fact that the glycolate oxidase in Charophytes, Xanthophytes, and *Cyanidium* participates in a modified glycolate pathway seem to give further support to this concept. However, the polyphyletic origin would not explain the sim-

Figure 3. Separation of organelles from a crude homogenate of *Cyanidium* on a linear flotation gradient. Enzyme activity: ordinate value × factor = nmoles per min per mL fraction. Citrate synthase, × 1; serine hydroxymethyltransferase, × 0.5; fumarase, × 1; catalase, × 10^3; glycolate oxidase, × 0.5; glutamate-glyoxylate aminotransferase, × 1; hydroxypyruvate reductase, × 1; serine-glyoxylate aminotransferase, × 0.04.

ilarities in the kinetic properties of glycolate pathway enzymes in *Cyanidium* and higher plants.

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