Mechanism of Glycolate Transport in Spinach Leaf Chloroplasts

TETSUKO TAKABE AND TAKASHI AKAZAWA
Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya
464, Japan

ABSTRACT

The incorporation of \(^{14}\text{CO}_2\) into glycolate by intact spinach leaf (\(\text{Spinacia oleracea} \) L. var. Kyoho) chloroplasts exposed to \(^{14}\text{CO}_2\) (Na\(\text{H}^{14}\text{CO}_3\), 1 millimolar) in the light was determined as a function of \(\text{O}_2\) concentrations in the reaction media. A hyperbolic saturation curve was obtained, apparent \(K_m\) (\(\text{O}_2\)) of 0.28 millimolar, indicating that glycolate is produced predominantly by ribulose-1,5-bisphosphate carboxylase/oxygenase. A concentration gradient of glycolate was invariably observed between chloroplast stroma and the outside media surrounding chloroplasts during photosynthetic \(^{14}\text{CO}_2\) fixation under an \(\text{O}_2\) atmosphere.

Glycolate transport into and out of chloroplasts was studied using the silicon oil centrifugation method. Both uptake and loss of glycolate were found to be rapid, with small temperature dependencies between 0°C and 25°C (\(Q_{10} = 1.1\)). The reaction rate as a function of the concentration of glycolate up to 30 millimolar was linear in both directions.

The effect of external pH on the reaction rate in both directions was also examined. Glycolate penetrates rapidly, even at pH 8, showing a surprisingly high permeation of the glycolate anion. This rate was about 30 micromoles per milligram chlorophyll per hour at 0°C, and the initial concentration of glycolate of 10 millimolar with a pH range of 7 to 8. The observed rate is comparable to the reported value for glycolate synthesis in chloroplasts under photosynthetic conditions. The uptake of glycolate into chloroplasts was accelerated below pH 7, while the rate of excretion was considerably lowered. It is, thus, suggested that undissociated glycolic acid penetrates the chloroplast envelopes more rapidly than does the anion.

Based on many recent investigations from various laboratories (7, 26–28), the basic mechanism of photosynthetic carbon oxidation cycle or glycolate pathway has been established, and it is generally accepted that three cell organelles, i.e., chloroplasts, peroxisomes, and mitochondria, are involved in the entire process (7, 26). However, little is known about the details of interorganellar transport of intermediates of the glycolate pathway, and the elucidation of the mechanism of metabolite transport among these organelles is urgently needed for a better understanding of photosynthesis.

Glycolate synthesis in chloroplasts through the oxidation of the Calvin-Benson cycle intermediates, and its subsequent excretion from chloroplasts constitutes the first step of the metabolic transport in photosynthesis. Two divergent views have been proposed concerning the formation of glycolate in chloroplasts. Asami and Akazawa (2) and Takabe et al. (23) recently suggested a modification of the original proposal by Gibbs (11) that glycolate originates from the 2-carbon transketolase intermediate. In the new proposal, oxidation would be by the superoxide radical (\(\text{O}_2^-\)), which is produced from monovalent reduction of \(\text{O}_2\) by PSI. Since \(\text{O}_2^-\) is found to be harmful to living systems, including chloroplast membranes (10, 18a), the scavenging effect of transketolase on \(\text{O}_2^-\) (second order rate constant between \(\text{O}_2^-\) and transketolase thiamine pyrophosphate-C\(_5\) complex, \(1 \times 10^8 \text{M}^{-1} \text{s}^{-1}\)) is possibly considered to have physiological significance. However, the maximum activity of glycolate production determined using the purified spinach transketolase was found to be low, about one order of magnitude lower than the one catalyzed by RuBP\(^2\) carboxylase/oxygenase at 20% \(\text{O}_2\) (23). In fact, it is the currently accepted view that the latter reaction occupies a more important role in producing glycolate. In this second mechanism, \(\text{P}\)-glycolate produced is supposed to be hydrolyzed by \(\text{P}\)-glycolate phosphatase, a specific phosphatase localized in chloroplasts (21, 22).

Glycolate is known to be excreted from chloroplasts (6, 17), algae (5, 25), blue green algae (8), and photosynthetic bacteria (3) under an \(\text{O}_2\)-containing atmosphere. Tolbert (26) has postulated that a glycogate/glyoxylate shuttle may operate in the photorespiratory process, but it has not been accepted, because glyoxylate-dependent \(\text{O}_2\) evolution is not observed in intact spinach chloroplasts (14). Although glycerate penetrates into intact chloroplasts of spinach as either the anion or the undissociated acid, no specific carrier for its transport has been demonstrated (13). Therefore, it seems likely that glycolate is also excreted from the chloroplasts by diffusion without the aid of a special carrier (cf. Ref. 12), but, so far, no conclusive experimental evidence in favor of this mechanism has been reported. The purpose of the work presented in this paper is to determine whether or not such a mechanism can be demonstrated as well as to study the properties of glycolate transport in chloroplasts.

MATERIALS AND METHODS

Isolation and Purification of Intact Chloroplasts. After grinding freshly harvested spinach leaves (\(\text{Spinacia oleracea} \) L. var. Kyoho) for 2 s in a Waring Blender, chloroplasts were separated by centrifugation in density gradients of silica sol Percoll (Pharmacia, Uppsala, Sweden), as reported previously by Takabe et al. (24). As demonstrated, the isolated intact chloroplasts were substantially pure (free from contamination of broken chloroplasts, peroxisomes, and mitochondria) and showed maximal photosynthetic \(^{14}\text{CO}_2\) fixation activities as high as 50–120 \(\mu\text{mol/mg Chl} \cdot \text{h}\).

Photosynthetic \(^{14}\text{CO}_2\) Fixation Under Various Concentrations of \(\text{O}_2\). The purified intact chloroplasts were suspended in a buffer solution containing 50 mm Hepes-NaOH (pH 8.0), 0.33 mm sorbitol, 1 mm MgCl\(_2\), 1 mm MnCl\(_2\), and 2 mm EDTA at 0.05 mg Chl/ml. The chloroplast suspension was flushed for 5 min in the dark with the appropriate gas atmosphere with the aid of Toray Model 104 NS \(\text{O}_2\) Pump (Toray Co. Ltd., Tokyo). The concentration of \(\text{O}_2\) dissolved in the suspension exposed to \(\text{O}_2\)-containing atmosphere was analyzed with a Rank Bros \(\text{O}_2\) Electrode (Cambridge, Eng-

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2 Abbreviation: RuBP, ribulose-1,5-bisphosphate.
land). After 3-min preillumination of the chloroplast suspension using a white tungsten lamp (20 Klux), NaH\(^{14}\)CO\(_3\) (final concentration of 1 mm, 5 mCi/mmol) was added, and the incubation continued for 3 min at 25°C. The procedures for determining total \(^{14}\)CO\(_2\) fixation, as well as the \(^{14}\)C incorporation into glycolate, were exactly the same as described previously by Asami et al. (4).

For the analysis of the incorporation of radioactivity into glycolate in the chloroplasts and in the medium, silicon-layer filtering centrifugation technique was used (see below).

**Transport Experiments.**

**Uptake of Glycolate.** Intact chloroplasts were suspended in a medium containing 50 mM Hepes-NaOH (pH 7.0), 0.33 mM sorbitol, 1 mM MgCl\(_2\), 1 mM MnCl\(_2\), and 2 mM EDTA at 0.3 mg Chl/ml. The reaction was started by adding \(^{14}\)C-glycolate of various concentrations (0.5 mCi/mmol) and incubated for various periods. Then, reaction was stopped by the silicon-oil-layer filtering centrifugation using a Beckman Microfuge B equipped with 0.4 ml polyethylene tubes, after the method of Klingenberg and Pfaff (19) and Heldt and Sauer (15). The tubes contained 20 \(\mu\)l 1 mM HClO\(_4\) at the bottom, above which was placed a layer of 50 \(\mu\)l silicon oil (AR20 [Wacker Chemie München]: 704 diffusion oil fluid [Dow Corning], 2:1). Then, 100 \(\mu\)l of chloroplast suspension was layered on top. In each experiment, the chloroplast space accessible to either tritiated water (\(\text{H}_2\text{O}\)) or \(^{14}\)C-sorbitol was determined following the method of Heldt and Sauer (15).

The quantity of glycolate produced in the sorbitol-permeable space was calculated from the glycolate concentration in the medium and subtracted from that found in the sedimented chloroplast preparation. The concentration of glycolate in the chloroplasts is related to the sorbitol-impermeable \(\text{H}_2\text{O}\) space (stroma space). The stroma space was approximately 33 \(\mu\)l/mg Chl (cf. 29 \(\mu\)l/mg Chl by Heldt and Sauer [15]).

**Excretion of Glycolate.** The intact chloroplasts at 1 mg Chl/ml were preincubated with varying concentrations of \(^{14}\)C-glycolate in a buffer of 50 mM Hepes-NaOH (pH 7.0) containing 0.33 mM sorbitol, 1 mM MgCl\(_2\), 1 mM MnCl\(_2\), and 2 mM EDTA for 15 min at 4°C. The whole sample was then diluted 10 times with the suspension buffer without glycolate and incubated for various periods; the reaction was then terminated by silicon-oil-layer filtering centrifugation, as described above. The measurements of glycolate transport in both directions (uptake and excretion) were carried out at 0°C in the dark, unless otherwise indicated.

**Reagents.** \([1-\text{\textsuperscript{14}}\text{C}]\)Glycolic acid (sodium salt) and \(\text{D}-[\text{\textsuperscript{14}}\text{C}]\)sorbitol were purchased from the Radiochemical Centre, Amersham, England, and \(\text{H}_2\text{O}\) (1 mCi/g), from New England Nuclear.

**RESULTS**

The formation of glycolate catalyzed by transketolase is supposed to be saturated at a relatively low concentration of O\(_2\), because the \(K_m\) for O\(_2\) production in PSII has been reported to be only 10 to 100 \(\mu\)m (1, 20). In order to examine the extent of glycolate production by this mechanism, the O\(_2\) dependency of glycolate formation in chloroplasts was examined (Fig. 1). The percentage of incorporation of \(^{14}\)CO\(_2\) into glycolate was determined under the gas atmospheres of varied partial O\(_2\) pressures. The concentration of O\(_2\) dissolved in the reaction media was determined by the O\(_2\) electrode. A hyperbolic saturation curve was observed, the apparent \(K_m\) for O\(_2\) being 0.28 mm. This result appears to support the currently accepted mechanism of the dominant role of RuBP carboxylase/oxygenase in the glycolate synthesis. O\(_2\)-dependent glycolate synthesis by a transketolase type mechanism, if functioning at low levels of free O\(_2\), would be limited in intact spinach chloroplasts. Therefore, it appears likely that most of the glycolate produced in chloroplasts is generated via P-glycolate.

Next, we examined whether or not glycolate is actually produced in chloroplasts during the photosynthetic \(^{14}\)CO\(_2\) fixation under 100% O\(_2\). In this experiment, the reaction was terminated by the silicon-oil-layer filtering centrifugation, and the radioactivity incorporated into the glycolate fraction was examined. The majority of the radioactivity was found in the external chloroplast fractions (Table I). Radioactivity was also detectable in the chloroplast fractions, and the concentration of glycolate inside the chloroplasts was found to be invariably higher than that in the outside medium. Glycolate is able to accumulate up to a concentration of 0.5 mM in the chloroplasts during 10-min photosynthesis under 100% O\(_2\), assuming the same specific radioactivity as that of NaH\(^{14}\)CO\(_3\) applied.

Time-course analyses of the rate of excretion and uptake of glycolate by chloroplasts were carried out, and the results are shown in Figure 2. The initial concentration of glycolate was 10 mm in the uptake experiment and 9 mm in the excretion experi-
Fig. 2. Uptake and excretion of glycolate by intact spinach chloroplasts. Experimental details are described in the text. In uptake experiments (A), 10 mM [14C]glycolate was used, and, in excretion experiments (B), chloroplasts were preliminarily incubated with 10 mM [14C]glycolate for 15 min at pH 7.0. In the latter system, the initial concentration of glycolate in chloroplasts determined by the silicon-oil-layer filtering centrifugation was 9 mM, and chloroplasts were diluted 10 times with a buffer of 50 mM Hepes-NaOH (pH 7.0) containing 0.33 mM sorbitol, 1 mM MgCl2, 1 mM MnCl2, and 2 mM EDTA. Reactions were terminated using silicon-oil-layer filtering centrifugation.

DISCUSSION

Experimental results obtained in the present investigation strongly suggest that glycolate molecules produced in chloroplasts appear to be excreted by diffusion without the aid of a specific carrier (cf. Ref. 12). If so, the rate of glycolate excretion from the organelle is determined by its concentration gradient between chloroplasts and cytoplasm. Therefore, it is likely that rapid oxidation of glycolate in peroxisomes and subsequent transformation are key parts of the overall process.

The present results show the significant effect of a pH gradient between chloroplasts and cytoplasm on glycolate transport. It must be recognized that almost all glycolate (pKa = 3.83, 25°C) in chloroplasts exists as the dissociated anion, especially under illumination, resulting in the alkalization of stromal pH (16). Therefore, to maintain the electroneutrality of stroma, it can be hypothesized that glycolate anions must be transported with either proton or ammonium ions (if available) or by the mechanism of anion exchange with glyceraldehyde or other permeable anions. It is, indeed, an important question whether or not glycolate can be transported by an anion exchange mechanism. Glyceraldehyde is an intermediate of the glycolate pathway and is assumed to move from the peroxisomes into chloroplasts (26). Heber et al. (13) reported that glyceraldehyde can penetrate the chloroplast envelopes as the dissociated anion and as the undissociated acid. Glycolate can penetrate rapidly even at pH 8, showing a surprisingly high permeation rate of the anion (Fig. 4). Although we could find a slight enhancing effect of glyceraldehyde in the medium on glycolate excretion from chloroplasts, much more defined experimental systems, such as inside-out vesicles of chloroplast envelopes, should be necessary for the elucidation of this mechanism. The detailed nature of the system should be clarified by future investigations.
FIG. 3. Concentration dependency of rates of uptake and excretion of glycolate. Reaction times were 15 s in uptake experiments (A) and 10 s in excretion experiments (B), both assayed at 0°C. In the latter experiments, the rate of excretion of glycolate was plotted against the internal concentration of glycolate. Other experimental details are given in Figure 2.

FIG. 4. Effect of external pH on glycolate uptake into or excretion from intact spinach chloroplasts. Experimental procedures were essentially the same as those given in Figure 3. Both the external and the initial concentrations of glycolate were 10 mM. The rate of glycolate transport was then measured in a suspending buffer of 50 mM Hepes-NaOH (varying pHs, 6.0 to 8.0) containing 0.33 mM sorbitol, 1 mM MgCl₂, 1 mM MnCl₂, and 2 mM EDTA.

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