THE INCORPORATION OF [^{18}O]OXYGEN INTO GLYCOLATE BY INTACT ISOLATED CHLOROPLASTS

G. H. LORIMER, G. H. KRAUSE and J. A. BERRY
Department of Environmental Biology, Research School of Biological Sciences, The Australian National University, Canberra, Australia

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1. Introduction

The mechanism of glycolate synthesis during photosynthesis is a subject of current interest [1–3]. A considerable body of evidence indicates that glycolate is synthesised primarily from ribulose-1,5-bisphosphate (RuBP), a reaction catalysed by RuBP carboxylase-oxygenase [4,5] which accounts for the incorporation of one atom of molecular oxygen into the carboxyl group of glycolate both in vivo [6,7] and in vitro [8]. However, two recent reviews [2,3] have implied that glycolate is synthesised by several different pathways without quantitatively assessing the contribution of these various pathways. In this report we establish that at least 80% of the glycolate produced by intact isolated chloroplasts is synthesised by reaction(s) which bring about the incorporation of one atom of molecular oxygen into the carboxyl group of glycolate.

2. Materials and methods

The methods used for the growth of spinach (*Spinacia oleracea* L., hybrid 102) and the isolation of intact chloroplasts have been previously described [9–11]. The chloroplasts used in this study were 87% intact as judged by the $K_3Fe(CN)_6$ method [12] and were capable of evolving O_2 in response to added bicarbonate (2 mM) at a rate of 90 μmol/h.mg chlorophyll (corrected for the presence of 13% broken chloroplasts).

Glycolate synthesis in the presence of [^{18}O]oxygen was conducted in an enclosed chamber containing 3.0 ml of a chloroplast suspension and 37.0 ml gas volume. The solution was stirred to facilitate gas exchange between the solution and gas phases. The chamber was thermostatted at 21°C, equipped with a Clark O_2 electrode and a capillary inlet system leading, via a needle valve, to the ion source of a DuPont 21-491B mass spectrometer. The gas phase consisted of argon and [^{18}O]oxygen (Norsk Hydro, Oslo, Norway) the concentration of which was determined by the O_2 electrode and the atom % ^{18}O by mass spectroscopy. Small samples of gas were introduced to the mass spectrometer and several scans from m/e 26–50 recorded at the beginning, end and several times during the course of the incubation. The ionising voltage was 15 eV and the temperature in the ion source was 225°C. From such scans the absolute enrichment of ^{18}O in the gas phase during the incubation was calculated using the formula $\%\ ^{18}O = (P_{36} + \frac{1}{2}P_{34})/(P_{32} + P_{34} + P_{36}) \times 100$, where P represents the peak heights of the ions at m/e 32, m/e 34 and m/e 36.

After 20 min incubation while illuminated by white light (500 nEinstein/cm^2 s), the chloroplast suspension was recovered and the chloroplasts removed by centrifugation. Glycolate was purified from the supernatant solution by means of ion-exchange chromatography and silylated by methods to be more fully described elsewhere. These methods are such that 96% of the ^{18}O in the carboxyl group of control preparations of [^{18}O]glycolate was retained.

Address for correspondence: G. H. Lorimer, Institut für Biochemie, Gesellschaft für Strahlen- und Umweltforschung, 8 München 2, Landwehrstr. 61, FRG

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Trimethylsilyl glycolate was separated by gas–liquid chromatography and its ^{18}O content was determined with a DuPont 21-491B mass spectrometer. The ^{18}O content of the glycolate was calculated from the spectra by the method of Biemann [13] using the values for the natural abundance of the constituent elements given by Beynon [14].

3. Results

The mass spectra of the trimethylsilyl glycolate shown in fig.1 contain 3 ions of interest at m/e 205, m/e 177 and m/e 161, the structures of which have been described by Petersson [15]. The ion at m/e 205 contains all three oxygen atoms, the ion at m/e 177 contains the hydroxymethyl oxygen atom and one of the carboxyl oxygen atoms and the ion at m/e 161 contains only the hydroxymethyl oxygen atom. Thus, analyses of these ions and their daughter ions at m/e-values 2 and 4 mass units higher, enables one to determine both the position and the quantity of ^{18}O incorporated.

As shown in fig.1b and 1c, no significant enhancement of the peaks at m/e 163 occurred when ^{18}O-labeled glycolate was prepared by exchange with [^{18}O]H_2O (1b) or by synthesis from $^{18}O_2$ (1c). This indicates that no ^{18}O is incorporated into the hydroxymethyl oxygens. The corrected intensity of m/e 205 (unlabeled), 207 (singly labeled) and 209 (double labeled) ions in the spectrum of glycolate prepared by exchange with water (1b) are consistent with an equal probability of incorporating ^{18}O into either carboxyl oxygen. In contrast nearly 70% of the glycolate molecules synthesised in the presence of $^{18}O_2$ are singly labeled and none are double labeled. This indicates that synthesis of glycolate in vivo by intact chloroplasts occurs by a non-random mechanism which incorporates a single atom of molecular oxygen into the carboxyl group. The synthesis of phosphoglycolate by purified RuBP carboxylase–oxygenase also results in this pattern of labeling which is distinctly different from exchange labeling [8].

In table 1 the absolute enrichments (atoms %) of the ^{18}O-labeled glycolate synthesized by intact chloroplasts is compared with the absolute enrichments of the [^{18}O]oxygen present in the gas phase. Regardless of the carbon source, whether CO_2 or triose phosphate, and regardless of the oxygen concentration (24% or 42%) present, the absolute enrichment of the

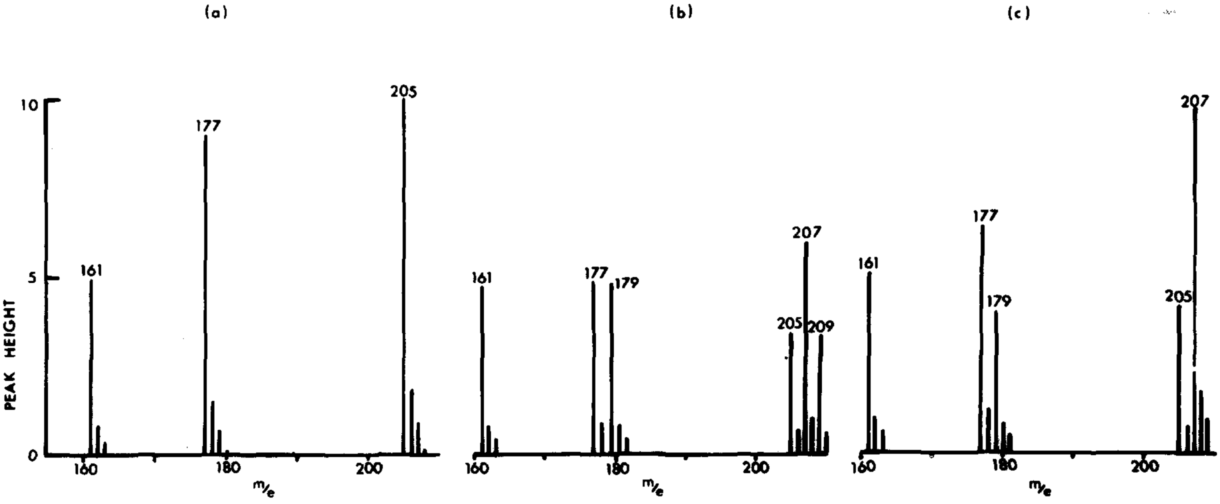

Fig.1. Mass spectra of (a) standard trimethylsilyl glycolate (b) trimethylsilyl glycolate prepared from glycolate which had been brought into isotopic equilibrium with [^{18}O]water (50.66 atom %) (c) trimethylsilyl glycolate prepared from glycolate synthesized by intact chloroplasts from CO_2 in the presence of an atmosphere of 24.7% oxygen (90.15 atom % ^{18}O) in argon. The trimethylsilyl glycolate was separated by gas–liquid chromatography (retention time 6 min) on a 2 m column of 3% SP-2250 on 80/100 Supelcoport (Supelco Inc., Penn., USA) at 80°C, flow rate 30 cc/min and introduced directly to the ion source of the mass spectrometer (DuPont 21-491B). Temperature of the ion source was 210°C and the ionizing voltage 70 eV.

Table 1
The absolute enrichment (atom %) of ^{18}O in the gas phase during synthesis and of glycolate synthesized by illuminated intact chloroplasts, and the final yield of ^{18}O-incorporation into glycolate

Substrate	% O_2	% O^{18} in O_2	% O^{18} in glycolate	% Yield
CO_2	24.7	90.2 ± 0.9	70.9 ± 1.3	78.6
CO_2	42.5	94.2 ± 0.6	78.8 ± 1.8	83.7
Triose-P	24.5	91.3 ± 0.7	73.5 ± 0.8	80.5

The basic medium contained 330 mM sorbitol, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid/NaOH buffer, pH 7.6, 10 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mMCl_2, 0.5 mM phosphate and about 1000 units/ml catalase. The substrate was either 1 mM CO_2 or 1.2 mM glycoaldehyde-3-phosphate (triose-P) and 8 units/ml of triose phosphate isomerase. The oxygen concentration during the incubation is given as percent of one atmosphere.

glycolate reaches a value some 80% of that of the oxygen with which it was synthesized. It follows that at least 80% of the glycolate synthesized by intact chloroplasts under these conditions is formed by reaction(s) resulting in the incorporation of one atom of molecular oxygen.

4. Discussion

In the experiments described above we have assumed that the absolute enrichment of the oxygen in the gas phase accurately reflects the absolute enrichment of the oxygen in the solution in which glycolate is synthesized. Owing to the photosynthetic production of [^{16}O]oxygen by the chloroplasts, the atom % ^{18}O must fall during illumination. Preliminary experiments using the Hoch/Kok inlet system [16], with which the isotope content of the dissolved gases is measured directly, indicate that there is considerable oxygen exchange and a substantial decrease in the % ^{18}O of the solution phase, even in the absence of any net oxygen evolution, as is the case during illumination of the chloroplasts with triose phosphate. Such exchanges would only slowly be registered in the gas phase, and would lead to the over-estimation of the absolute enrichment of the oxygen in the solution. Also, small losses of ^{18}O, by exchange (< 4%) occur during isolation and derivization of the labeled glycolate. The value of 80% relative enrichment must therefore be regarded as a minimum value. Incorporation of a single atom of molecular oxygen into phosphoglycolate by isolated, purified RuBP carboxylase–oxygenase has been demonstrated. Until other glycolate synthesizing reactions are established as bringing about such labeling of glycolate, we must conclude that this reaction catalysed by RuBP carboxylase-oxygenase accounts for most (> 80%) of the glycolate produced during photosynthesis.

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