Comparison of the Kinetic Properties of Ribulose Biphosphate Carboxylase in Chloroplast Extracts of Spinach, Sunflower and Four Other Reductive Pentose Phosphate-Pathway Species

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Extracts from chloroplasts of spinach, sunflower and four other reductive pentose phosphate (C_3)-pathway species were measured spectrophotometrically with or without a modified preactivation procedure. In all six species this modification yielded K_m (CO_2) values in the range of 7–15 μM and maximum velocities, at 20°C, of 129–431 μmol of CO_2 carboxylated/h per mg of chlorophyll. In general, both the carboxylation and electron-transport capacities of sunflower were somewhat greater than that of the other species, and this is discussed in relation to the superior rates of photosynthesis believed to be displayed by the parent tissue.

Ribulose biphosphate carboxylase (EC 4.1.1.39) has posed many problems since it was first characterized in the 1950's (Quayle *et al.*, 1954). It is an important enzyme; by far the greater part of all CO_2 fixed by green plants is incorporated in the reaction that it catalyses. The evidence in support of the reductive pentose phosphate (C_3) pathway (Benson-Calvin cycle), in which it plays a central role (Calvin & Bassham, 1962), is compelling. However, from the outset, there have been doubts about its ability to fulfil its proposed function (Peterkofsky & Racker, 1961). These have centred on what was originally believed (Weissbach *et al.*, 1956) to be its extraordinarily low affinity for CO_2 [K_m (HCO_3^-) 11 mM], although it is evident that maximal velocity is as important in this context as affinity.

A precise value of the carboxylase activity required *in vivo* is not easily arrived at because of the many assumptions that must be made. However, it may be calculated (Heath, 1969; Lilley & Walker, 1975) that a difference of about 200 p.p.m. of CO_2 between the external atmosphere and the site of carboxylation would permit an influx of CO_2 at a sufficient rate to maintain fixation at the value achieved by the average plant under favourable conditions in its natural environment [about 100 μmol of CO_2 fixed/h per mg of chlorophyll (Rabinowitch, 1956)]. In this regard spinach performs like the average species (Sawada & Miyachi, 1974; Lilley & Walker, 1975) and so far as we can judge (see, e.g., Heath, 1969; Whittingham, 1974) a concentration of CO_2 at the carboxylation site of approx. 100 p.p.m. would be acceptable to many physiologists. A rate

of 100 μmol of CO_2 fixed/h per mg of chlorophyll at a concentration of 100 p.p.m. thus provides a yardstick against which the performance of the isolated enzyme may be measured. During the last decade, work on activation by Mg^{2+} of the carboxylase [for a review see Walker (1973), and also Bahr & Jensen (1974), Andrews *et al.* (1975) and Lorimer *et al.* (1976)] has diminished the apparent inadequacy of carboxylation rates to such an extent that the enzyme at last seems equal to its task *in vivo* (Lilley & Walker, 1975). In retrospect it appears that many of the earlier problems may have arisen from the assay of the enzyme in an inactive, or partly activated, state. The present paper reports a modification of the activation procedure used in the spectrophotometric assay of Lilley & Walker (1974) and shows that in this assay the carboxylase then displays essentially the same characteristics as those described by Lorimer *et al.* (1976). In addition, the characteristics of carboxylase from five other species have been examined. Of these, sunflower was selected because it is reported to photosynthesize at rates that compare favourably with some dicarboxylate (C_4)-pathway species, despite being handicapped by photorespiration (El-Sharkaway & Heskestad, 1964; Warren-Wilson, 1966; Rajan *et al.* 1973). The reason for the high photosynthetic performance of sunflower is not known, but if it is to do with the relative efficiency of the photosynthetic apparatus then comparative values of carboxylase activity would clearly be of interest. Moreover, high rates of photosynthesis have been obtained with a reconstituted chloroplast system from sunflower (Delaney & Walker, 1976), thereby encouraging the belief that the enzymes concerned could be recovered from this species in good yield.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Material and Methods

Plant material

Spinach (*Spinacia oleracea*) U.S. Hybrid 424, sunflower (*Helianthus annuus*) var. Mammoth and sugar beet (*Beta vulgaris*) were grown in water culture as previously described (Lilley & Walker 1974, 1975).

Peas (*Pisum sativum*) var. Feltham First were grown in trays in a vermiculite/compost mixture and were used when the shoot had reached a height of 5–7 cm. Lettuce (*Lactuca sativa*) var. Balloon was germinated in trays of compost and used when 8–10 cm in size.

Good King Henry (*Chenopodium bonus henricus*) was grown in the departmental garden and harvested in early summer when the plants were in full leaf.

Chemicals and enzymes

Analytical grade reagents were used wherever possible. Solutions of creatine kinase (from rabbit muscle) and mixed 3-phosphoglycerate kinase/NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (from yeast) were prepared daily from freeze-dried solid [all biochemicals were from Sigma (London) Chemical Co., Kingston upon Thames KT2 7BH, U.K.]

Media

All media used in the preparation of intact chloroplasts contained sorbitol (0.33 M), together with the additives listed below.

Grinding medium (spinach). This contained 10 mM- $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM- MgCl_2 and 2 mM-sodium isoascorbate at pH 6.5. For sugar beet and Good King Henry 0.1% bovine serum albumin was also added.

Grinding medium (sunflower and lettuce). This was as used by Delaney & Walker (1976), and contained 10 mM- $\text{Na}_4\text{P}_2\text{O}_7$, 5 mM- MgCl_2 , 2 mM-sodium isoascorbate, 1 mM-EDTA (disodium salt), 1 mM-sodium thioglycollate, 10 mM- NaCl , 1% bovine serum albumin and 1% Carbowax-20 [poly(ethylene glycol), mol.wt. 20000] at pH 8.0.

Grinding medium (peas). This contained 50 mM- Na_2HPO_4 , 50 mM- KH_2PO_4 , 5 mM- MgCl_2 , 0.1% (w/v) NaCl and 0.2% (w/v) sodium isoascorbate at pH 6.5.

Washing medium. Each 100 ml contained 5 mM- MgCl_2 and 4 ml of grinding medium (spinach). For all species other than spinach 0.1% bovine serum albumin was also added.

Resuspending medium. This contained 1 mM- MnCl_2 , 1 mM- MgCl_2 , 2 mM-EDTA and 50 mM-Hepes adjusted to pH 7.6 with KOH.

Extraction medium. This was 1 ml of resuspending medium in 25 ml of water containing dithiothreitol (3 mM).

Chloroplasts and chloroplast extract were pre-

pared as before (Cockburn *et al.*, 1968; Walker, 1971; Lilley *et al.*, 1974). For purposes of assay the stromal protein equivalent to 5 μg of chlorophyll was calculated from the volume of the supernatant (chlorophyll extract) and the chlorophyll content of the pellet (Lilley *et al.*, 1974, 1975). The chlorophyll contributed by ruptured chloroplasts (see under 'Chloroplast intactness') was disregarded in this calculation, i.e. on the basis of earlier observations (Lilley *et al.*, 1975), the stromal protein was assumed to be derived only from those chloroplasts that were intact in the ferricyanide assay.

Assay of ribulose biphosphate carboxylase

The standard procedure was based on the spectrophotometric assay of Lilley & Walker (1974). CO_2 -free mixtures were prepared as follows in boiled deionized water. Solutions containing 0.66 M-sorbitol, 100 mM-Hepes, 20 mM- KCl , 2 mM-EDTA and 30 mM- MgCl_2 were flushed with N_2 for 10 min at pH approx. 4.0 and then adjusted to pH 7.9, under N_2 , with CO_2 -free KOH. These solutions were then stored under N_2 , and used together with CO_2 -free water, in the preparation of assay mixtures.

Each assay mixture (final vol. 1 ml) contained 0.33 M-sorbitol, 50 mM-Hepes, 10 mM- KCl , 1 mM-EDTA, 15 mM- MgCl_2 , 0.26 mM-NADH, 5 mM-phosphocreatine, 5 mM-dithiothreitol, 5 mM-ATP, 2 units of creatine kinase (EC 2.7.3.2), 15 units of phosphoglycerate kinase (EC 2.7.2.3), 5 units of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), 10 mM- NaHCO_3 (or appropriate concentration) and chloroplast extract equivalent to 5 μg of chlorophyll. After incubation in a water bath at 20°C for 5 min the reaction was started by the addition of 0.5 μmol of ribose 5-phosphate. The change in A_{340} was followed in a Pye-Unicam SP.800 recording spectrophotometer at 20°C and the rate was expressed as μmol of CO_2 /per mg of chlorophyll.

Activation

Where indicated the standard carboxylase assay was preceded by activation of the enzyme as follows. Extract (100 μl) was placed in a small plastic tube with 2 μl of 1 M- NaHCO_3 and 2 μl of 1 M- MgCl_2 to give a final concentration of 20 mM- HCO_3^- and 20 mM- Mg^{2+} . The tube was then incubated in a water bath at 20°C for 5 min. A sample of activated extract (equivalent to 5 μg of chlorophyll) was then added to a cuvette containing the other components of the assay mixture and the reaction was started by the addition of ribose 5-phosphate (0.5 μmol).

In calculating the substrate (HCO_3^-) concentration, allowance was made for the amount of NaHCO_3 carried over with the activated extract to the assay mixture and the amount of HCO_3^- present in the ' CO_2 -free' buffer and water.

Measurement of HCO_3^- concentration in 'CO₂-free' water or buffer

HCO_3^- present in 'CO₂-free' buffer was measured by injecting a small sample of the buffer through a Suba-seal into 1–2 ml of rapidly stirred 1 M-HCl in a small glass tube. The CO₂ released was detected by an i.r. gas analyser (Analytical Development Co., Hoddesden, Herts. EN11 0AQ, U.K.). The system was calibrated by the addition of portions of freshly made NaHCO₃ solution.

Chloroplast intactness

This was calculated from the rates of ferricyanide-dependent O₂ evolution (Lilley *et al.*, 1975) measured with a Clark-type oxygen electrode (Delieu & Walker, 1972) purchased from Hansatech, King's Lynn, Norfolk, U.K.

Determination of protein:chlorophyll ratio

Chloroplast suspension (50 μl) was added to 10 ml of aq. 80% (v/v) acetone in a conical glass centrifuge tube and shaken. After centrifugation at 10000g for 5 min the supernatant was decanted for chlorophyll measurement. The protein pellet was washed once with 80% acetone and resuspended in 1 ml of 0.1 M-NaOH followed by 4 ml of biuret reagent (Layne, 1957). After 10 min of colour development the A_{550} was measured in a Pye-Unicam SP.800 spectrophotometer against a reagent blank. The cell position nearest to the photomultiplier was used because of slight turbidity caused by insoluble components of the chloroplast membranes. A 0.2% bovine serum albumin standard was used routinely to check a previously prepared concentration curve.

Statistical analysis and method of calculation

The kinetic data were analysed by using an ALGOL program for the computer-assisted calculation of enzyme-kinetic data (Hoy & Goldberg, 1971), which is based on the statistical method of Wilkinson (1961). The program utilizes a weighted non-linear fit of the data and provides the s.e.m. of the estimates as well as all the information needed to construct the Lineweaver-Burk plot. A feature of the program is that the calculation process recycles to ensure that the parameter shifts are well within their s.e.m. values.

The concentration of dissolved CO₂, the form of inorganic carbon used by the ribulose biphosphate carboxylase (Cooper *et al.*, 1969), was calculated from pH and bicarbonate concentration by using the Henderson-Hasselbach equation, with a value of 6.39 for the pK' at 20°C, of the CO₂-hydration reaction (Umbreit *et al.*, 1972). The rate of CO₂ assimilation within the chloroplast was calculated from the V_{max} and K_m (CO₂) values of the isolated enzyme assuming a stromal CO₂ concentration of

4.5 μM (the corresponding value for air-saturated water at 20°C is about 12 μM).

Results

Ribulose biphosphate carboxylase is most conveniently assayed in a linked enzyme system in which the immediate product, 3-phosphoglycerate, is reduced to triose phosphate (Racker, 1962; Anderson & Fuller, 1969). In the presence of an ATP-generating system the reduction is quantitative and the decrease in A_{340} associated with the oxidation of NADH in the triose phosphate dehydrogenase reaction gives an easy and meaningful measure of carboxylase activity (Lilley & Walker, 1974). Since this method was first published, however, improvements have been made in the pre-activation procedure (Andrews *et al.*, 1975; Lorimer *et al.*, 1976). In agreement with these results

Fig. 1. Plots of velocity against substrate concentration for ribulose biphosphate carboxylase activity of sunflower chloroplast extract, measured by (a) the original or (b) the modified method

The carboxylase was preincubated for 5 min at 20°C in the reaction mixture minus ribose 5-phosphate (Δ) or the enzyme was separately preincubated for 5 min at 20°C, in 20 mM-NaHCO₃ before addition of ribose 5-phosphate (●).

Fig. 2. Double-reciprocal plots of the data from Fig. 1 for ribulose biphosphate carboxylase

we now find that activation is best achieved by pre-incubation with 20 mM-NaHCO₃/20 mM-MgCl₂. At 15–20°C, activation is normally complete in 5–6 min. Over a 5 min period full activation was not achieved at temperatures below 15°C, but there was no further improvement between 15 and 30°C. The change in the preincubation procedure was without appreciable effect on the V_{max} , but decreased the apparent K_m (HCO₃⁻) by a factor of 4 in spinach and by a larger value in sunflower.

Fig. 1 illustrates this difference in an experiment with stromal protein from sunflower. Fig. 2 is a double-reciprocal plot of the same data. The apparent K_m (HCO₃⁻) and V_{max} values from Fig. 2 were 2.38 mM and 385 μmol/h per mg of chlorophyll in the Lilley & Walker (1974) assay (▲) and 0.23 mM and 335 μmol/h per mg measured by the same method together with the new activation procedure (●). The results of several more experiments are summarized in Table 1. If a lowered K_m is taken as a measure of more effective activation, the new activation procedure led to a slightly more active spinach carboxylase than that used by Badger & Andrews (1974) and by Andrews *et al.* (1975). Nevertheless, the apparent K_m (CO₂) values for the sunflower carboxylase were even lower (on average, some 30% below those for spinach).

Table 1. K_m and V_{max} values for ribulose biphosphate carboxylase extracted from six C₃-pathway plants. Results are means with the numbers of preparations used in parentheses.

Species	K_m (HCO ₃ ⁻) (mM)	K_m (CO ₂) (μM)	V_{max} (μmol of CO ₂ /h per mg of chlorophyll)
Good King Henry (2)	0.415 ± 0.078	12.4 ± 2.3	214.5 ± 5.0
Sugar beet (5)	0.285 ± 0.036	8.52 ± 1.06	169.2 ± 3.2
Lettuce (3)	0.402 ± 0.026	12.1 ± 0.77	303.9 ± 3.4
Pea (3)	0.329 ± 0.047	9.9 ± 1.4	228.9 ± 5.4
Spinach (6)	0.370 ± 0.050	11.1 ± 1.5	273.5 ± 6.7*
Sunflower (6)	0.260 ± 0.044	7.76 ± 1.3	342.6 ± 9.7

* Considerably higher values have been previously recorded for spinach in our laboratory (see, e.g., Lilley & Walker, 1975).

Table 2. Comparison of electron transport and ribulose biphosphate carboxylase activity in a variety of C₃-pathway species. Electron transport was measured as uncoupled ferricyanide-dependent O₂ evolution, and carboxylase activity within the chloroplast was calculated from the K_m (CO₂) and V_{max} (Table 1) by using the Michaelis-Menten equation and assuming a concentration of CO₂ of 100 p.p.m. in the gas phase, at 20°C. Results are means (with range in parentheses).

Species	Rate of electron transport (μmol of O ₂ /h per mg of chlorophyll)	Rate of carboxylation (μmol of CO ₂ /h per mg of chlorophyll)	No. of experiments
Spinach*	229 (188–287)	81 (44–147)	6
Sunflower	366 (294–472)	128 (83–158)	6
Good King Henry	211 (185–237)	60 (40–80)	2
Sugar beet	161 (137–184)	62 (47–74)	5
Lettuce	236 (206–287)	84 (64–105)	3
Pea	285 (257–304)	74 (37–99)	3

* In previous work (Lilley *et al.*, 1975) the mean value for electron-transport rate in 82 separate chloroplast preparations was 273 μmol of O₂ evolved/h per mg of chlorophyll.

For purposes of comparison, a survey was also made of the apparent K_m (CO_2) in several C_3 -pathway species from which it was possible to obtain reasonably intact chloroplasts. The results are also summarised in Table 1. The K_m (CO_2) values ranged from 7 to 15 μM , and were therefore very similar to those recorded for spinach and mostly higher than those obtained with sunflower.

Finally (in Table 2) measured electron-transport rates are recorded together with the rates of carboxylation that would occur within the stroma if the characteristics of the activated carboxylase were those displayed *in vivo* and if the stromal $[\text{CO}_2]$ was equivalent to 100 p.p.m. in the gas phase. Here again there is an indication that the rate of electron transport in sunflower chloroplasts may be somewhat better than that of the other species examined. The calculated carboxylation rates are in the same range, or a little lower, than those which would be displayed by the parent tissues in air.

Discussion

The above results show that relatively small adjustments to the preincubation procedure [along the lines suggested by Lorimer *et al.*, (1976)] change the characteristics that ribulose biphosphate carboxylase displays in the spectrophotometric assay of Lilley & Walker (1974). The apparent K_m (CO_2) is decreased by a factor of approx. 4 to give values close to 11 μM , whereas the V_{max} was largely unchanged. [It may be noted that the V_{max} values recorded for the spinach carboxylase during the course of the present work were somewhat low. A maximum of 947 $\mu\text{mol/h}$ per mg of chlorophyll has been previously reported, and measured values in the range of 800–1000 have been recorded on a number of occasions in this laboratory since the present work was completed (P. McNeil, unpublished work).] As before (Lilley & Walker, 1975) the spinach carboxylase would be more or less equal to its task *in vivo*, i.e. it would be capable, at 20°C, of displaying rates of approx. 100 μmol of CO_2 fixed/h per mg of chlorophyll (see Table 2) given CO_2 at the concentration that might obtain within the functioning chloroplast (Lilley & Walker, 1975). The behaviour of the carboxylase from three other C_3 -pathway species was very similar. On the basis of the existing measurements the affinity of the sunflower enzyme for CO_2 was marginally superior (some 30%) to that from spinach, but in view of the nature of the measurements and the total scatter for all species examined (7–15 μM) we would be reluctant to attach much significance to this aspect. Moreover, it is by no means certain that the best activation and assay procedures have yet been devised. Thus the characteristics displayed by

the sunflower enzyme *in vitro* could either reflect its true behaviour within the plant or be simply a more ready response to the activation procedures used in the present work. In the latter case the possibility of making meaningful comparisons between the carboxylation potential of different species must remain remote. It is of interest, in this context, that there is a greater difference between spinach and sunflower extracts in relation to their response to the new activation procedure.

What does seem clear is that a ribulose biphosphate carboxylase, with characteristics broadly similar to that of the spinach enzyme, can be extracted from several higher-plant species in quantities roughly commensurate with the rates of photosynthesis that these species might be expected to display in air at 20°C. In itself this is hardly remarkable, but it must be borne in mind that until recently the recoverable carboxylase activity had seemed entirely inadequate for its postulated task *in vivo* (see the introduction).

Whether the new activation procedures will eventually permit more detailed correlations between carboxylation capacity and whole-plant photosynthesis remains to be established. Many species contain high concentrations of phenolics, or other compounds, which interfere with chloroplast isolation, and comparisons between the maximal velocities of carboxylases from different sources could become little more than an index of the ease of extraction. Nevertheless some of the highest rates of carboxylation and electron transport recorded in the present work were displayed by sunflower fractions, despite the fact that active chloroplasts are not easily isolated from this species (Delaney & Walker, 1976).

As stressed above, the apparently high affinity for CO_2 of the sunflower carboxylase might be questioned at the present time, because of current uncertainties about the effectiveness of the activation procedures. On the other hand, the measurement of uncoupled electron transport to ferricyanide (which was incidental to this investigation) is a widely used and simple procedure which has been carried out routinely in this laboratory for some years. Although (at 20°C, and in red light at an irradiance of 300 W/m^2) we have occasionally recorded electron-transport rates for spinach chloroplasts as high as 300 μmol of O_2/h per mg of chlorophyll, rates of over 400 (as displayed by sunflower) have previously been outside our experience. For spinach chloroplasts in saturating CO_2 , photosynthesis is not limited by the carboxylation potential, but by electron transport (Lilley & Walker, 1975). The present results suggest that the sunflower has at least the same capacity for carboxylation as spinach, and almost certainly a more efficient electron-transport system. Both factors would be expected to contribute to the known superiority of its photosynthetic performance.

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