Volume 260, number 1, 62–66 FEB 08009 January 1990

Substrate isomerization inhibits ribulosebisphospate carboxylase-oxygenase during catalysis

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Received 5 October 1989; revised version received 17 November 1989

The inhibition of purified spinach ribulosebisphosphate carboxylase-oxygenase which occurs progressively during catalysis in vitro is caused by accumulation of at least two tight-binding inhibitors at the catalytic site. Reduction of these inhibitors with NaB^3H_4, followed by dephosphorylation, produced a mixture of xylitol and arabinitol, thus identifying one of them as D-xylulose 1,5-bisphosphate. It was formed during carboxylation, presumably by a stereochemically incorrect reprotonation of the 2,3-enediolate intermediate bound at the catalytic site. Under the conditions used, this epimerization occurred approximately once for every 400 carboxylation turnovers. Another inhibitor may be 3-keto-D-arabinitol 1,5-bisphosphate which would also be formed by misprotonation of the enediolate intermediate, but at C-2 rather than at C-3.

Ribulosebisphosphate carboxylase-oxygenase; Ribulose 1,5-bisphosphate carboxylase-oxigenase, D-; Enzyme mechanism; Enzyme inhibition; Xylulose 1,5-bisphosphate, D-; Photosynthesis

1. INTRODUCTION

Rubisco catalyzes the initial reactions of the photosynthetic carbon reduction cycle and its photorespiratory appendage, the glycolate pathway [1]. The purified enzyme from higher plants becomes progressively inhibited during catalysis [2–9] because a strongly inhibitory phosphorylated compound accumulates on the catalytic site [6,7,9]. Cyanobacterial [10] and algal [8] Rubiscos display the phenomenon to a much smaller extent, if at all. With the higher-plant enzyme, inhibition is promoted by acidic pH and low substrate CO_2 concentrations [6,7]. While Rubisco requires carbamylation on an active site lysine residue, followed by the binding of a divalent metal ion, for activity [1], it remains fully carbamylated during inhibition [6,7]. The inhibitor may be released by acid denaturation of inhibited Rubisco and used to inhibit fresh enzyme. The inhibitor is a slow, tight-binding inhibitor and its binding is characterized by strong negative cooperativity [6,7]. Its apparent average K_D (neglecting the negative cooperativity) is approximately 0.2 μM [6,7]. Here we show that at least two compounds are involved in this inhibition. We identify one of them as xylulose-P_2 and show that it is produced as a by-product of the Rubisco reaction. Some of these data were included in a presentation to the VIIIth International Congress on Photosynthesis (Stockholm, 1989) [6].

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Abbreviations: ribulose-P_2, D-ribulose 1,5-bisphosphate; xylulose-P_2, D-xylulose 1,5-bisphosphate; 3-ketoarabinitol-P_2, 3-keto-D-arabinitol 1,5-bisphosphate; carboxyarabinitol-1-P, 2′-carboxy-D-arabinitol 1-phosphate; Rubisco, ribulose-P_2 carboxylase-oxygenase; glycerol-P, glycerol-3-phosphate

2. MATERIALS AND METHODS

2.1. *Materials*

Ribulose-P_2 was prepared without exposure to pH above 7 and Rubisco was purified from spinach leaves, both as previously described [7]. Rubisco concentrations were measured spectrophotometrically [11]. Xylulose-P_2 (Li^+ salt) was kindly provided by J. Pierce (Du Pont). Aldolase and glycerol-P dehydrogenase, both from rabbit muscle, were obtained from Boehringer and desalted before use by diafiltration against 50 mM Hepes-NaOH, pH 8.2, containing 5 mM dithiothreitol.

2.2. *Synthesis of inhibitor*

Fully carbamylated Rubisco (19 μM protomers) was allowed to consume completely 6.3 mM ribulose-P_2 in the presence of 4 mM Bicine-NaOH, pH 8.0, 20 mM $MgCl_2$, 2.8 mM $NaHCO_3$, 0.4 mM EDTA at 23°C. Acid produced during the reaction was neutralized by progressive addition of Na_2CO_3, which also maintained the HCO_3^- concentration constant. Acid production ceased after approximately 10 min and after a further 10 min, HCl was added to a final concentration of 0.1 M. Little, if any, precipitation of Rubisco occurred. Therefore, denatured protein was removed by centrifugation of the solution through an Amicon MPS-1 apparatus using a YMT membrane. The filtrate contained the inhibitor and was stored frozen without neutralization.

2.3. *Identification of inhibitor*

Inhibitor was synthesized as described in section 2.2, except that the incubation after acid production stopped was prolonged to 2 h to

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00145793/90/$3.50

ensure that no trace of ribulose-P_2 remained. The inhibitor bound to Rubisco was then isolated from the reaction mixture by gel filtration through Sephadex G-50 fine (Pharmacia) equilibrated with 10 mM Tris-acetate, pH 8.0. The high-molecular-mass fraction was collected and adjusted to pH 5.0 with acetic acid, followed by addition of 4 vols of methanol. After several hours storage at −80°C, the precipitated protein was removed by centrifugation and the methanol removed by rotary evaporation. The residue, in water, was treated with 1 ml of Dowex 50-X8 (H^+ form) to remove cations and then dried in vacuo. The residue was dissolved in 40 μl of a solution containing 0.6 M Tris-acetate, pH 8.6, and 37 mM NaB^3H_4 (Amersham, 32000 cpm/nmol) which was prepared immediately before use. A control, containing 15 nmol of ribulose-P_2, and a blank, containing no sample, were treated similarly. After 30 min at 23°C, the solutions were diluted, passed over 1 ml columns of Dowex 50-X8 (H^+ form), and evacuated to dryness. The residues were dissolved in 1 ml of 0.1 M Tris-acetate, pH 8.4, and applied to a 1.1 × 17 cm column of Dowex 1-X8 (Cl^- form), equilibrated with 3 mM HCl. The column was developed with a NaCl gradient as shown in fig.1. Fractions representing the latest eluting peak, which was absent in the blank, were pooled, adjusted to pH 9 with Tris base, made 1 mM in $MgCl_2$, 0.1 mM in Zn acetate and 0.05 mM in EDTA, and incubated overnight at 23°C with 3 μg/ml of bovine alkaline phosphatase (Boehringer). The pH was then adjusted to 10 and the radioactivity adsorbed to 1 ml columns of Affi-gel 601 (Bio-rad), equilibrated with 0.1 M NH_4OH. The columns were washed successively with 0.1 M NH_4OH and water and the radioactivity was finally eluted with 0.2 M formic acid. The formic acid eluates were passed through 0.2 ml columns of Dowex 50-X8 (H^+ form), evacuated to dryness, and the residues dissolved in 12 μl of 90% (v/v) ethanol containing 9 μg each of ribitol, arabinitol and xylitol. These samples were then spotted on silica gel thin layers (Merck) which were developed twice with propan-2-ol/ethyl acetate/water, 83:11:6 (by vol.). After drying, the plates were scanned with a radiochromatogram scanner (Berthold), followed by visualization of the standard pentitols with alkaline permanganate [12] (fig.2).

2.4. *Measurement of xylulose-P_2*

Aliquots of inhibitor-containing solutions, prepared as described in section 2.2, were added to a solution which ultimately contained, in a final volume of 1 ml, 0.2 M Hepes-NaOH, pH 7.5, 50 μM NADH and 40 μg of rabbit-muscle glycerol-P dehydrogenase. The absorbance at 340 nm was measured and the final extent of the decrease in absorbance which followed the further addition of 0.21 mg of rabbit-muscle aldolase reflected the xylulose-P_2 concentration. No absorbance change occurred if the dehydrogenase or the inhibitor sample was omitted. The same assay system was also used to detect any xylulose-P_2 present in ribulose-P_2 preparations. Because ribulose-P_2 competitively inhibits aldolase, the aldolase and glycerol-P dehydrogenase concentrations were increased to 1.0 and 0.16 mg/ml, respectively. Aldolase also has a weak reactivity towards ribulose-P_2 [13] which causes a slow constant rate of absorbance decline which continues after the 'burst' due to xylulose-P_2. This was compensated for by extrapolating the slow final rate back to the time of addition of aldolase. These difficulties caused the assay to be less sensitive when ribulose-P_2 was present but, by adding xylulose-P_2 to the assays, it was shown that a xylulose-P_2 contamination equal to 0.05% of the ribulose-P_2 could be detected with confidence.

2.5. *Inhibition of Rubisco by inhibitor preparations*

Aliquots of inhibitor preparations, prepared as described in section 2.2, were incubated with glycerol-P dehydrogenase, NADH and, where indicated, aldolase for 16 min at 25°C, then added to solutions containing fully carbamylated Rubisco and incubated for a further 60 min before addition of ribulose-P_2 for a 1 min assay. Final concentrations were: Bicine-NaOH (pH 8.2), 75 mM; $MgCl_2$, 24 mM; bovine serum albumin, 0.1% (w/v); NADH, 0.1 mM; $NaH^{14}CO_3$ (2000 cpm/nmol), 10 mM; ribulose-P_2, 0.4 mM; rabbit muscle glycerol-P dehydrogenase, 70 μg/ml; rabbit muscle aldolase, 180 μg/ml; Rubisco, 0.35 μM protomers. Reactions were stopped by adding formic acid to 16% (v/v), dried, and acid-stable radioactivity measured by scintillation counting. Where indicated, the inhibitor preparation was adjusted to pH 12.4 with NaOH and, after 2 min, readjusted to pH 1.5 with HCl before use.

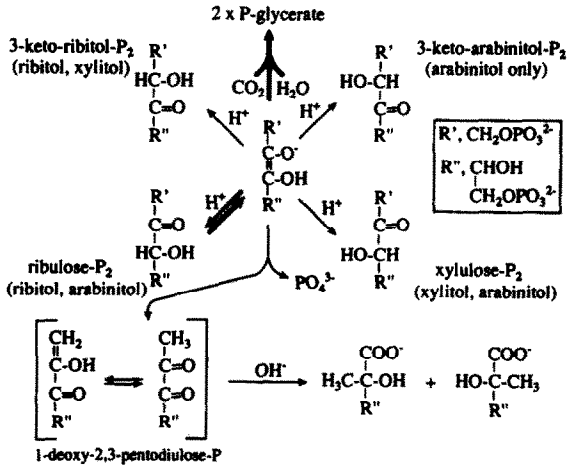
3. RESULTS AND DISCUSSION

3.1. *A strategy for identifying the inhibitor*

Substrate CO_2 does not become incorporated into the inhibitor [6,7]. Therefore, if the intermediate is produced as an abortive by-product of the Rubisco reaction, it must be derived from an intermediate prior to the formation of the carboxylated intermediate. This leaves only one candidate, namely, the 2,3-enediolate intermediate produced by abstraction of the C-3 proton from ribulose-P_2. It is quite a likely candidate. It could be subject to misprotonation, either stereochemically incorrectly at C-3, or at C-2 rather than C-3, which would produce various pentulose bisphosphates which are quite likely to be strong inhibitors. Indeed, one of them, xylulose-P_2, shows inhibitory characteristics [14] which strongly resemble those of the inhibitor which accumulates during catalysis [6,7]. Another possible route for inhibitor production might be by β-elimination of the C-1 phosphate group, as has been shown to occur with the enediolate intermediate in solution [15,16]. These possibilities are summarized in scheme 1. Since all of the putative inhibitors are ketones, reduction with tritiated borohydride provides a means of labelling them as a prelude to identification.

3.2. *Identification of an inhibitor*

The commercial NaB^3H_4 preparations used in this study were contaminated with labelled, acid-involatile materials of unknown origin. These contaminants could be separated from the products of reduction of the inhibitor or ribulose-P_2 by anion-exchange chromatography (fig.1). The ratio between the two



Scheme 1. Possible fates for the enediolate intermediate. The bold arrows indicate the carboxylation reaction sequence. The other reactions are abortive side reactions leading to potential inhibitors. The products expected after reduction and dephosphorylation of the four possible pentulose bisphosphates are shown in parentheses.

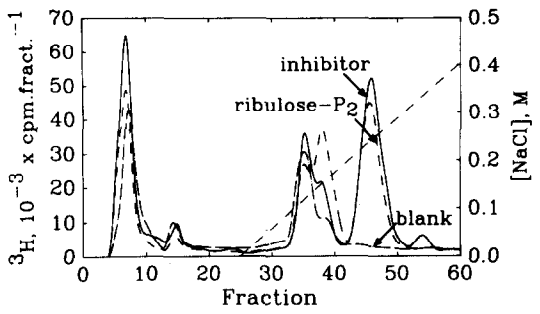


Fig.1. Anion-exchange chromatography of the products of NaB^3H_4 reduction of an inhibitor preparation, ribulose-P_2 and a blank reaction containing no sample. See section 2.3 for methods.

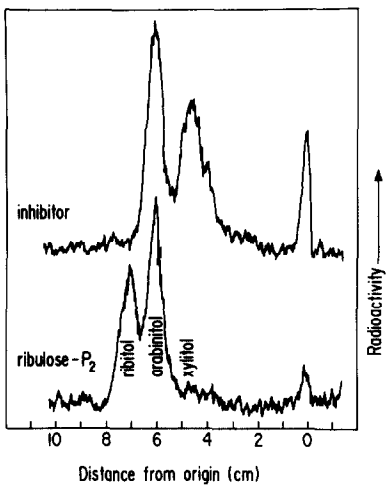


Fig.2. Radiochromatogram scans of thin-layer chromatograms of the material from the latest eluting peak in fig.1 after dephosphorylation. The positions shown for the pentitols were determined by cochromatography with standards. The origin peak in both samples varied in size in different experiments. It may reflect oxidative degradation of the pentitols during sample application or trailing of earlier eluting components into the bisphosphate peak during anion-exchange chromatography. See section 2.3 for methods.

contaminant peaks which eluted in fractions 33–40 varied between experiments, even in the blank containing no sample, and no significance should be attached to differences in these ratios. The reduced forms of the inhibitor and ribulose-P_2 were more anionic than the contaminants and no peak in this position was apparent in the blank. Furthermore, identical peaks were obtained for the inhibitor and ribulose-P_2. Therefore, we conclude that the inhibitor must be a bisphosphate. After dephosphorylation with alkaline phosphatase, thin-layer chromatography resolved the material in this strongly anionic peak into two components (fig.2). When the starting material was ribulose-P_2, ribitol and arabinitol were observed, as expected, whereas for the inhibitor, the products were arabinitol and xylitol. Inspection of scheme 1 shows that this combination of pentitols could only arise if the inhibitor preparation contained xylulose-P_2. However, the presence of some 3-ketoarabinitol-P_2, whose reduction and dephosphorylation would produce arabinitol only, cannot be excluded. Stereochemical bias in the reduction of pentulose bisphosphates by borohydride [17], which could produce unequal amounts of arabinitol and xylitol from xylulose-P_2 alone, precludes calculation of the potential contribution of 3-ketoarabinitol-P_2 to the observed peak area ratio.

3.3. *Xylulose-P_2 is a catalytic by-product*

Since xylulose-P_2 is an excellent substrate for aldolase [18], it may be measured using the coupled spectrophotometric assay described in section 2.4. Xylulose-P_2 was readily detected in inhibitor preparations produced as described in section 2.2, at a level equal to 0.26% of the ribulose-P_2 originally present (89% of the Rubisco protomer concentration). Moreover, the assay did not detect the presence of any xylulose-P_2 in the starting ribulose-P_2 preparation above the detection limit of 0.05% of the ribulose-P_2 present. Therefore, a minimum of 80% of the xylulose-P_2 ultimately found must have been produced during the consumption of the ribulose-P_2 catalyzed by Rubisco. Even when 0.6 mg/ml of aldolase, 40 μg/ml of glycerol-P dehydrogenase and 30 μM NADH were included during consumption of ribulose-P_2 as described in section 2.2, xylulose-P_2 was still detected in the acidified filtrate, although at a reduced level (0.11% of the initial ribulose-P_2 or 36% of the Rubisco protomer concentration). Apparently, the aldolase system was able to scavenge any xylulose-P_2 which was released from Rubisco's active site (and any traces of xylulose-P_2 which may have been present in the starting ribulose-P_2) but it was unable to access that fraction of the xylulose-P_2 which remained bound at the active site. This observation is consistent with the lack of effect of the xylulose-P_2 scavenging system on the kinetics of slow inhibition [6,7].

3.4. *Another inhibitor as well as xylulose-P_2*

When the inhibitor preparation was exposed to aldolase as described in section 2.5, its inhibitory capacity was partially destroyed (fig.3). This effect was not due to the presence of phosphatases in the aldolase preparation. When ribulose-P_2, at a concentration similar to that expected for the inhibitor (0.4 μM final concentration), was carried through a procedure similar to that described in section 2.5, except that Rubisco was not present during the initial 76 min incubation period with aldolase and glycerol-P dehydrogenase but was added in excess subsequently to carboxylate all of the ribulose-P_2 remaining, more than 97% of the ribulose-P_2 supplied initially was carboxylated. Therefore, the levels of phosphatases active against ribulose-P_2 and, by inference, xylulose-P_2 must have been insignificant.

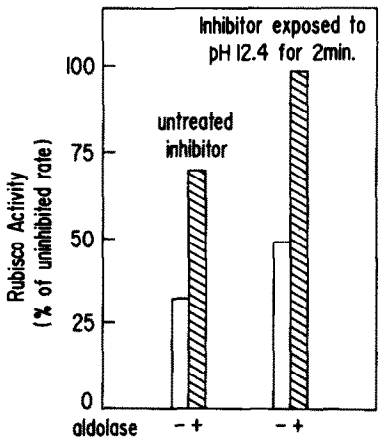


Fig.3. Effect of aldolase and alkali treatment on the inhibitory capacity of inhibitor preparations. The uninhibited turnover rate (100%) was 1.6 s^{-1}. See section 2.5 for methods.

Not only was the reduction in inhibitory capacity caused by aldolase only partial but exposure of the preparations to pH 12.4 for 2 min also partially relieved the inhibition (fig.3). The alkaline treatment had virtually no effect on authentic xylulose-P_2. Its half-time for decay was shown, using the enzymatic assay, to be several hours at pH 12.4. Treatment of the inhibitor preparation with alkali followed by aldolase was required to completely destroy inhibitory potency. Therefore, at least two inhibitors must be present in the inhibitor preparations: one is xylulose-P_2, which is sensitive to aldolase but not very sensitive to alkali; the other is resistant to aldolase but quickly destroyed by alkali.

We speculate that the alkali-labile inhibitor might be one or both of the 3-pentulose bisphosphates formed by protonation of the enediolate intermediate at C-2, rather than at C-3 (scheme 1). Such compounds might be labile by virtue of the acidity of the protons α to the carbonyl group and the tendency for the adjacent phosphate dianion to abstract that proton, as has been established with the analogous triose phosphate, glyceraldehyde 3-phosphate [19]. For these symmetrical 3-pentulose bisphosphates, either the C-2 or the C-4 proton would be abstracted to produce enediolates identical or analogous to the enediolate intermediate. In solution, these enediolates rapidly eliminate the C-1 phosphate group as shown in scheme 1 [15,16]. 3-Ketoarabinitol-P_2 is a particularly attractive candidate for the second inhibitor because it would be formed by attack of a proton on the enediolate intermediate from the same direction that CO_2 and O_2 attack from in the carboxylation and oxygenation reactions. This direction would be expected to have some exposure to solvent that would facilitate proton attack. Alternatively, it is possible that the alkali-labile inhibitor is 1-deoxy-2,3-pentodiulose 5-phosphate, formed by β-elimination of the enediolate on the active site (scheme 1). Alkali-induced rearrangement [15] would provide a ready explanation for its alkali lability. However, it is difficult to imagine how this 5-monophosphate could bind as tightly to Rubisco as the inhibitor does [6,7]. Furthermore, treatment of inhibitor preparations with *o*-phenylene diamine or H_2O_2, both of which should react with this dicarbonyl compound, did not greatly reduce their inhibitory capacity [7].

If the second inhibitor is 3-ketoarabinitol-P_2, then, as mentioned in section 3.2, its reduced and dephosphorylated product would simply have contributed to the arabinitol peak in fig.2. However, if the second inhibitor is the analogous ribitol epimer, then some labelled ribitol should have been produced, or if it is the dicarbonyl compound mentioned above, then a labelled monophosphate should have appeared. Neither was detected and this strengthens the case for 3-ketoarabinitol-P_2 being the second inhibitor. However, the second inhibitor's instability may have led to its loss during the work-up prior to borohydride reduction and, therefore, we do not consider that we have conclusively established the presence of 3-ketoarabinitol-P_2. A different approach will be required to identify the second inhibitor convincingly.

3.5. *Implications for Rubisco's mechanism*

Derivation of the inhibitors from the enediolate intermediate is consistent with the greater extent of inhibition observed at low CO_2 concentration [6,7] when the fraction of Rubisco in the enediolate form is greatest [20]. This also suggests an explanation for the lesser susceptibility of the algal [8] and cyanobacterial [10] enzymes to this kind of inhibition. An obvious way for the higher-plant Rubisco to have evolved towards its superior catalytic potency (higher k_{cat}/K_m ratio [1]) would be by altering the equilibrium between ribulose-P_2 and the enediolate intermediate on the active site in favour of the enediolate. This would increase the steady-state concentration of the intermediate species with which CO_2 directly reacts. Any abortive side reactions to which the enediolate may be susceptible would also increase but this might be an acceptable price to pay for improved catalytic effectiveness. The greater extent of inhibition at low pH [6,7] is also consistent with the inhibitors being produced by abortive misprotonation reactions.

3.6. *Physiological implications*

It is not likely that such severe inhibition of Rubisco could be tolerated in the chloroplast during active photosynthesis. Recently, it has been shown that Rubisco activase prevents and reverses this kind of inhibition [9] and is also able to cause the dissociation from the active site of another strong inhibitor, carboxyarabinitol-1-P, which accumulates nocturnally in some plants [21]. Presumably, activase is able to

catalytically effect a temporary change in Rubisco's active site topography which loosens the binding of such inhibitors, although the mechanism by which activase achieves this is presently unknown. Since activase is regulated by light via the stromal ATP/ADP ratio [22], it is possible that, when the activity of activase is suppressed at low light levels, inhibitory pentulose bisphosphates accumulate on Rubisco's active site, thereby modulating its activity to an appropriate level. Thus these inhibitors may play a regulatory role analogous to that of carboxyarabinitol-1-P, particularly for species which lack carboxyarabinitol-1-P, such as spinach. Of course, once removed from Rubisco, further mechanisms would be required to metabolize the inhibitors. In the case of xylulose-P_2, stromal aldolase could perform this task. However, additional means for disposing of glycolaldehyde phosphate would then be necessary. Alternatively, a phosphatase specific for the C-1 phosphate, similar or perhaps identical to fructose bisphosphatase or sedoheptulose bisphosphatase, would return this inhibitor to the chloroplast's metabolism as xylulose 5-phosphate.

REFERENCES

[1] Andrews, T.J. and Lorimer, G.H. (1987) in: Photosynthesis (Hatch, M.D. and Boardman, N.K. eds) The Biochemistry of Plants, Vol.10, pp.131–218, Academic Press.

[2] Andrews, T.J. and Hatch, M.D. (1971) Phytochemistry 10, 9–15.

[3] Laing, W.A. and Christeller, J.T. (1976) Biochem. J. 159, 563–570.

[4] Sicher, R.C., Hatch, A.L., Stumpf, D.K. and Jensen, R.G. (1981) Plant Physiol. 68, 252–255.

[5] Mott, K.A. and Berry, J.A. (1986) Plant Physiol. 82, 77–82.

[6] Andrews, T.J., Badger, M.R., Edmondson, D.L., Kane, H.J., Morell, M.K. and Paul, K. (1989) in: Progress in Photosynthesis Research (Baltscheffsky, M. ed.) Kluwer, Dordrecht, in press.

[7] Edmondson, D.L., Badger, M.R. and Andrews, T.J., submitted.

[8] Yokota, A. and Kitaoka, S. (1989) Plant Cell Physiol. 30, 183–191.

[9] Robinson, S.P. and Portis, A.R. (1989) Plant Physiol. 90, 968–971.

[10] Andrews, T.J. and Ballment, B. (1984) Proc. Natl. Acad. Sci. USA 81, 3660–3664.

[11] Paulsen, J.M. and Lane, M.D. (1966) Biochemistry 5, 2350–2357.

[12] Hay, G.W., Lewis, B.A. and Smith, F. (1963) J. Chromatogr. 11, 479–486.

[13] Gray, G.R. and Barker, R. (1970) Biochemistry 9, 2454–2462.

[14] McCurry, S.D. and Tolbert, N.E. (1977) J. Biol. Chem. 252, 8344–8346.

[15] Paech, C., Pierce, J., McCurry, S.D. and Tolbert, N.E. (1978) Biochem. Biophys. Res. Commun. 83, 1084–1092.

[16] Pierce, J., Andrews, T.J. and Lorimer, G.H. (1985) J. Biol. Chem. 261, 10248–10256.

[17] Lorimer, G.H., Gutteridge, S. and Reddy, G.S. (1989) J. Biol. Chem. 264, 9873–9879.

[18] Mehler, A.H. and Cusic, M.E. (1967) Science 155, 1101–1103.

[19] Richard, J.P. (1984) J. Am. Chem. Soc. 106, 4926–4936.

[20] Jaworowski, A., Hartman, F.C. and Rose, I.A. (1984) J. Biol. Chem. 259, 6783–6789.

[21] Robinson, S.P. and Portis, A.R. (1988) FEBS Lett. 233, 413–416.

[22] Robinson, S.P. and Portis, A.R. (1988) Plant Physiol. 86, 293–298.