BBA 65549

STUDIES ON THE ACTIVE SITE OF THE ENZYME RIBULOSE-DIPHOSPHATE CARBOXYLASE

GEORGE AKOYUNOGLOU, JOAN-HEOS ARGYROUDI-AKOYUNOGLOU AND H. METHENITOU
Nuclear Research Center "Democritus", Greek Atomic Energy Commission, Athens (Greece)

(Received June 16th, 1966)

SUMMARY

Ribulosediphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39, formerly known as carboxydismutase), the enzyme catalyzing the CO_2 fixation reaction in photosynthesis, forms a complex with CO_2 that can be isolated by passage through an ion-exchange column. By using diazomethane to stabilize the $^{14}CO_2$–enzyme complex, digestion with trypsin (EC 3.4.4.4) to hydrolyze it, and ion-exchange column chromatography to separate the resulting peptides, it has been possible to obtain a highly radioactive peptide. Moreover, the isolated radioactive peptide is slightly colored, which raises the question as to the nature and function of the chromophore.

The radioactive peptide can be further hydrolyzed by pepsin (EC 3.4.4.1) or carboxypeptidase (EC 3.4.2.1). Separation of the pepsin hydrolysate by ion-exchange column chromatography gave only one radioactive fraction, which was also colored. The ultraviolet absorption spectrum of this fraction depends greatly on the pH of the solution. Resolution of the carboxypeptidase hydrolysate by paper chromatography gave one radioactive spot and fourteen ninhydrin-positive spots. Experiments with ^{14}C-labeled δ-aminolevulinic acid showed that after separation of the tryptic peptides of ribulosediphosphate carboxylase, radioactivity was present in the colored peptide.

INTRODUCTION

Previous studies on the mechanism of action of ribulosediphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39, formerly known as carboxydismutase), the enzyme catalyzing the carboxylation of ribulose 1,5-diphosphate during photosynthetic CO_2 reduction, have shown that the reaction proceeds in two or probably three steps with the enzyme-bound CO_2 (ribulosediphosphate carboxylase–CO_2 complex) as an intermediate. The ribulosediphosphate carboxylase–CO_2 complex has been isolated by passage through a Dowex 1-X8 anion-exchange column. The formation of the complex required the presence of magnesium ions[1]. The

ribulosediphosphate carboxylase reaction has been thought to involve the following steps:

$$Enzyme + Mg^{2+} + CO_2 \rightleftharpoons (Enzyme\text{–}Mg^{2+})\text{–}CO_2 \quad (1)$$

$$(Enzyme\text{–}Mg^{2+})\text{–}CO_2 + \text{ribulose 1,5-diphosphate} \rightleftharpoons [(Enzyme\text{–}Mg^{2+})\text{–}CO_2]\text{–ribulose 1,5-diphosphate} \quad (2)$$

$$[(Enzyme\text{–}Mg^{2+})\text{–}CO_2]\text{–ribulose 1,5-diphosphate} \xrightarrow{H_2O} Enzyme + Mg^{2+} + 2\ \text{3-phospho-D-glyceric acid} \quad (3)$$

The detailed arrangement of the six carbon atoms represented on the right of Eqn. 2 above is not known. The stability and/or concentration of this quadruple complex must be very low.

Recently[2] it was suggested that the –SH group of cysteine is the active site of the enzyme where ribulose 1,5-diphosphate is bound, and that one step in the overall enzymatic reaction is the hemimercaptole formation between ribulose 1,5-diphosphate and –SH group, which in turn reacts with the enzyme-bound CO_2 to give 3-phospho-D-glyceric acid. This conclusion was based on the observation that ribulose 1,5-diphosphate protects the enzyme against iodoacetamide inhibition. However, more recent investigations in our laboratory[3] have shown that other compounds, such as sugar phosphates, carbamyl phosphate, and sulfate and phosphate ions, can protect the enzyme against iodoacetamide inhibition, which indicates another interpretation of the data.

The site of CO_2 attachment on the enzyme has not yet been elucidated. It is of interest to attempt to find the active site of the enzyme on which CO_2 is bound, and to elucidate the overall dismutation reaction. This paper describes the stabilization of the $^{14}CO_2$–enzyme complex by diazomethane, the digestion of the stabilized complex with trypsin (EC 3.4.4.4), the separation of the tryptic peptides, and the isolation of a highly radioactive peptide, which is also colored. The hydrolysis of the radioactive peptide by pepsin (EC 3.4.4.1) and carboxypeptidase (EC 3.4.2.1), the separation of the hydrolysates, and the isolation of one radioactive compound as yet unidentified, are also reported.

METHODS

Preparation of ribulosediphosphate carboxylase

Ribulosediphosphate carboxylase was obtained from the chloroplasts of spinach leaves (*Spinacea oleracea*). The chloroplasts were first isolated as described earlier[4], and then lysed osmotically with 0.01 M phosphate buffer (pH 7.4) for more than 2 h to yield the chloroplast extract. The chloroplast extract was made 90% saturated with respect to ammonium sulfate and left for 24 h at 2°. The pH of the solution was adjusted to 7.0 by the addition of conc. NH_4OH. The precipitated protein was collected by centrifugation at 55 000 × *g* for 15 min, redissolved in 0.001 M Tris buffer (pH 8.0) and dialyzed for 24 h against 0.001 M Tris buffer (pH 8.0) containing 0.05 mM EDTA. The protein was then fractionated by repeated ammonium sulfate precipitation. The enzyme solution was left for at least 2 h at 2° at each ammonium sulfate level, and the precipitate formed was collected by centrifugation at 55 000 × *g* for 15 min. The fraction precipitated in the range 31–35.5% $(NH_4)_2SO_4$ contained the enzyme ribulosediphosphate carboxylase and was used for the experiments. The enzyme was stored as a precipitate in 50% satd. $(NH_4)_2SO_4$ solution (pH 7.0) at 2°.

Before each experiment a small quantity of the precipitate was dissolved in 0.001 M Tris buffer (pH 8.0) containing 0.05 mM EDTA, and dialyzed against the same buffer for 24 h. Protein concentration was measured by the absorbance at 280 mμ on a Beckman DK-2A spectrophotometer; calculations were done on the basis of the relationship $A_{280\ m\mu} \times 0.6 =$ mg protein per ml, which is based on the colorimetric determination of the protein concentration by the Folin phenol reagent[5].

The ribulosediphosphate carboxylase assay

The activity of the enzyme was measured by determining the radioactivity of the 3-phosphoglyceric acid formed as acid-stable radioactivity after incubation of the enzyme with $MgCl_2$, ribulose 1,5-diphosphate and ^{14}C-labeled bicarbonate. Each reaction mixture contained 20 μmoles of Tris–HCl buffer (pH 8.0), 2.5 μmoles of $MgCl_2$, 30–40 μg of enzyme, 1.5 μmoles of ^{14}C-labeled bicarbonate (specific activity, 5.5 μC/μmole), and 0.09 μmole of ribulose 1,5-diphosphate in a final volume of 0.2 ml. All components were mixed at 0°, and immediately after the addition of ribulose 1,5-diphosphate the reaction mixture was incubated at 25° for exactly 10 min. The reaction was stopped by the addition of 0.2 ml of 5% acetic acid in methanol. An aliquot of the mixture was spread on a thin aluminum planchet with the aid of a micropipette, dried under an infrared lamp and counted in a Low Beta Baird Atomic gas-flow counter.

Preparation of the ribulosediphosphate carboxylase–Zn^{2+} complex

The ribulosediphosphate carboxylase–Zn^{2+} complex was prepared as follows. The enzyme was precipitated by 2.5 mM $ZnSO_4$, the precipitate was separated by centrifugation and dissolved in 0.1 M cysteine (pH 7.4). The dissolved enzyme was reprecipitated by an equal volume of a 100% satd. $(NH_4)_2SO_4$ solution (pH 7.0), the precipitate was dissolved in 0.01 M phosphate buffer (pH 7.4), and dialyzed against 0.01 M Tris buffer (pH 8.2) for 24 h. Experiments done with $^{65}Zn^{2+}$ have shown that after this treatment zinc ions are still bound to the enzyme.

Preparation of the $^{14}CO_2$–ribulosediphosphate carboxylase complex

The $^{14}CO_2$–ribulosediphosphate carboxylase complex was prepared as previously described[1], except that each reaction mixture contained either 20 mg of enzyme or 20 mg of the enzyme–Zn^{2+} complex. The recovery of the complex from the Dowex 1-X8 column was 85%. Immediately after the isolation the $^{14}CO_2$–ribulosediphosphate carboxylase complex was treated with a freshly prepared ethereal solution of diazomethane. The stable methylated complex was concentrated almost to dryness and then digested with trypsin.

Preparation of diazomethane

Diazomethane was prepared from Ni-nitroso-*p*-toluolsulfomethylamide as previously described[6].

Tryptic digestion

The enzyme or the methylated $^{14}CO_2$–enzyme complex was digested with trypsin at 37° for 48 h, at pH 7.8 at the end of which 75% hydrolysis had occurred. A ratio of trypsin to ribulosediphosphate carboxylase 1:20 (w/w) in 1 M Tris buffer was used.

The reaction was stopped by the addition of an equal volume of 3% trichloroacetic acid. The trichloroacetic acid-soluble fraction of the tryptic digest was lyophilized and dissolved in 5 ml of pyridine–collidine–acetic acid buffer (10, 10 and 0.2 ml, respectively, in 1000 ml), pH 8.2. In some experiments the trichloroacetic acid-soluble fraction of the tryptic digest was first dialyzed against a 4-fold excess of 0.01 M Tris buffer (pH 8.2) for 24 h, and then the diffusible as well as the undiffusible peptides were lyophilized and dissolved in 5 ml of the pyridine–collidine–acetic acid buffer.

Separation of the tryptic peptides

A Dowex 1-X2 ion-exchange column was used for the separation of the tryptic peptides. Dowex 1-X2 (100–200 mesh, acetate form) was washed and equilibrated with a pyridine–collidine–acetic acid buffer (10, 10, and 0.06 ml, respectively, in 1000 ml), pH 8.8, and then packed into a 1 cm × 150 cm column. The column was loaded with the mixture of the soluble peptides and then eluted step-wise at room temperature with pyridine–collidine–acetic acid buffer (10, 10 and 0.2 ml, respectively, in 1000 ml) pH 8.2, 0.03 M acetate containing 1% collidine and 1% pyridine (pH 7.3), 0.02 M acetate, 0.2 M acetate, 0.35 M acetate, 0.50 M acetate, 0.65 M acetate, 1.7 M acetate, and 5.0 M acetate[7]. The eluted fractions (3.5 ml) were collected with a Beckman Model 132 automatic fraction collector and analyzed by the Folin–Lowry[8] method or by the ninhydrin test[9]. An aliquot was also spread on a thin aluminum planchet with the aid of a micropipette, dried under an infrared lamp and counted on a Low Beta Baird Atomic gas-flow counter.

Pepsin hydrolysis

The radioactive peptide was digested with twice-crystallized pepsin in a ratio of pepsin to peptide of about 1:40 (w/w) at pH 1.8 for 3 h. The pH was maintained constant in an autotitrator. After digestion the mixture was neutralized, applied to the 1 cm × 150 cm Dowex 1-X2 column and eluted by the usual procedure.

Carboxypeptidase hydrolysis

A fraction of the radioactive peptide was also digested with carboxypeptidase at pH 8.0 for 8 h, according to FRAENKEL-CONRAT, HARRIS AND LEVY[10]. The carboxypeptidase digest was resolved by paper chromatography with water-saturated phenol containing 0.1% ammonia, or butanol–acetic acid–water (4:4:5, v/v) as solvents.

Experiment with [4-^{14}C]δ-aminolevulinic acid

Young spinach leaves with stems (25 pieces cut from *Spinacea oleracea* plants grown in our laboratory)were each placed separately in small tubes containing 0.2 ml of an aqueous solution of [4-^{14}C]δ-aminolevulinic acid (1.2 μC per 10 μg per ml), and then exposed to continuous illumination (white light from fluorescent tubes). After 2 h the leaves were cleaned, transferred to tubes containing water and left in the light for 24 h. Ribulosediphosphate carboxylase was prepared from the crude extract of the so treated spinach leaves by ammonium sulfate fractionation. The crude extract was obtained by homogenizing the leaves, with stems and midribs removed, in a Waring blendor with 0.01 M Tris buffer (pH 8.0) containing 1 mM EDTA, and then centrifuging the homogenate. The isolated enzyme was digested with trypsin, and the tryptic peptides were separated by the procedure described above.

Trypsin, twice crystallized, was a product of Koch and Light Co. (England); pepsin, twice crystallized, was from Worthington, N.J.; carboxypeptidase, twice crystallized, was a product of Nutritional Biochemicals, Cleveland, Ohio; N-nitroso-*p*-toluolsulfomethylamide was from Fluka (Switzerland); [4-^{14}C]δ-aminolevulinic acid was purchased from the department of radioisotopes of CEA, Gif-Sur-Yvette (France); ^{14}C-labeled bicarbonate was prepared from barium [^{14}C]carbonate, a product of New England Corp., Boston, Mass.

RESULTS

Preliminary experiments showed that if ribulosediphosphate carboxylase were digested with trypsin, and the trichloroacetic acid-soluble tryptic peptides were separated on a Dowex 1-X2 ion-exchange column, fourteen major peptides were obtained. Fig. 1 shows the peptide elution pattern. One of the 14 peptides was distinctly

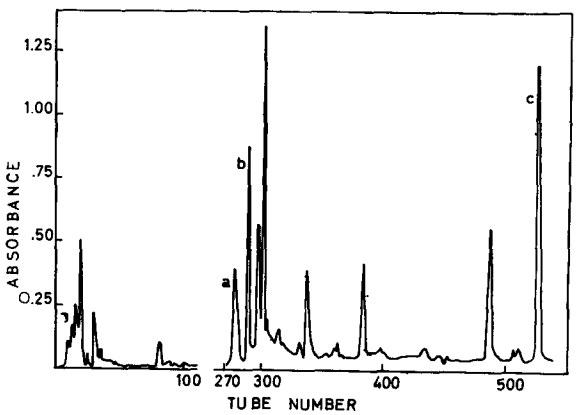

Fig. 1. Chromatography on a Dowex 1-X2 column (1 cm × 150 cm) of the tryptic peptides of a 105-mg sample of ribulosediphosphate carboxylase. 'Absorbance' stands for the integrated absorbance at 500 mμ of the Folin color obtained per half hour with 1 ml (of 3.5 ml) eluate after being completely dried. The column was equilibrated with pyridine–collidine–acetic acid buffer (10, 10 and 0.06 ml, respectively, in 1000 ml) and eluted by means of: (1) 1% collidine, 1% pyridine, $6.25 \cdot 10^{-3}$ M acetic acid (pH 8.2); (2) 1% collidine, 1% pyridine, 0.03 M acetic acid (pH 7.3); (3) 0.02 M acetic acid; (4) 0.2 M acetic acid; (5) 0.35 M acetic acid; (6) 0.5 M acetic acid; (7) 0.65 M acetic acid; (8) 1.7 M acetic acid; and (9) 5 M acetic acid. Buffers were changed at 73, 196, 275, 315, 360, 412, 464, and 510 fractions respectively. Elution was performed at room temperature.

yellow (peptide c), and two others only slightly yellow (peptides a and b). Approximately the same chromatographic pattern was obtained with the tryptic digest of the $^{14}CO_2$–ribulosediphosphate carboxylase complex, stabilized by diazomethane. Fig. 2 shows the peptide elution pattern as well as the radioactivity pattern of the methylated complex. A comparison of Fig. 1 and 2 shows a slight difference in the chromatographic pattern, due probably to methylation. The radioactivity pattern shows that from all the peptides eluted only one is highly radioactive, namely that eluted at the 29th fraction after the last change of eluant to 5 M acetic acid (peptide c), thus showing that the $^{14}CO_2$ binding to the enzyme is highly specific, as one would expect for

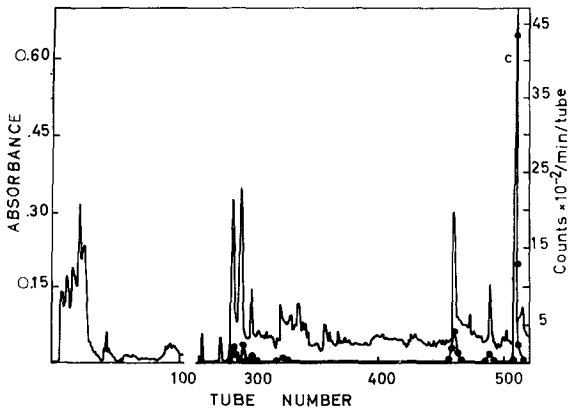

Fig. 2. Chromatography on a Dowex 1-X2 column (1 cm × 150 cm) of the tryptic digest of 70 mg $^{14}CO_2$–ribulosediphosphate carboxylase complex stabilized by diazomethane. The enzyme was treated with Zn^{2+} as described in the experimental section. Elution and other conditions as in Fig. 1. Buffers were changed at 75, 185, 256, 293, 330, 370, 443, and 485 fractions. ———, absorbance at 500 mμ of the Folin color obtained per half hour with 1 ml (out of 3.5 ml) eluate after being completely dried. ●—●, ^{14}C radioactivity per fraction.

a binding to the active site. The same peptide is also the one that is distinctly yellow. This peptide contained 45% of the radioactivity applied to the column, the loss probably being due to slow hydrolysis. However, it is evident that methylation by diazomethane is effective in stabilizing the $^{14}CO_2$–ribulosediphosphate carboxylase complex.

As shown in Figs. 3a and 3b, the radioactive peptide is barely diffusible from the dialysis tubing in 24 h. Complete hydrolysis of the radioactive peptide by autoclaving a lyophilized fraction at 118° for 23 h with constant-boiling HCl (5.7 M), causes the loss of all the ^{14}C radioactivity. Separation of the hydrolysate by two-dimensional paper chromatography (water-saturated phenol containing 0.1% ammonia, and butanol–acetic acid–water, 4:1:5, v/v, as solvents) showed 14 ninhydrin-positive spots.

The chromatographic and radioactivity pattern of the pepsin hydrolysate of the radioactive peptide c showed only one radioactive peptide containing 70% of the radioactivity applied to the column. This peptide was eluted as the same elution volume at which the peptide not treated by pepsin was recovered, namely in the 29th fraction after the change of the eluant to 5 M acetic acid, and it was also yellow. However, the possibility that hydrolysis of the peptide occurred in such a way that the overall charge did not change is not excluded, since smaller Folin-positive peaks have been obtained, and since the ultraviolet spectrum of the peptide changed considerably after pepsin treatment.

The spectral characteristics of the peptide are greatly dependent on the pH of the solution as shown in Figs. 4a and 4b. Fig. 4a is the ultraviolet spectrum of the pepsin-treated peptide as eluted from the ion-exchange column. Fig. 4b shows the ultraviolet spectrum of the same peptide in HCl and NaOH.

Separation of the carboxypeptidase hydrolysate of the radioactive peptide c by paper chromatography and autoradiography showed only one radioactive spot as yet unidentified.

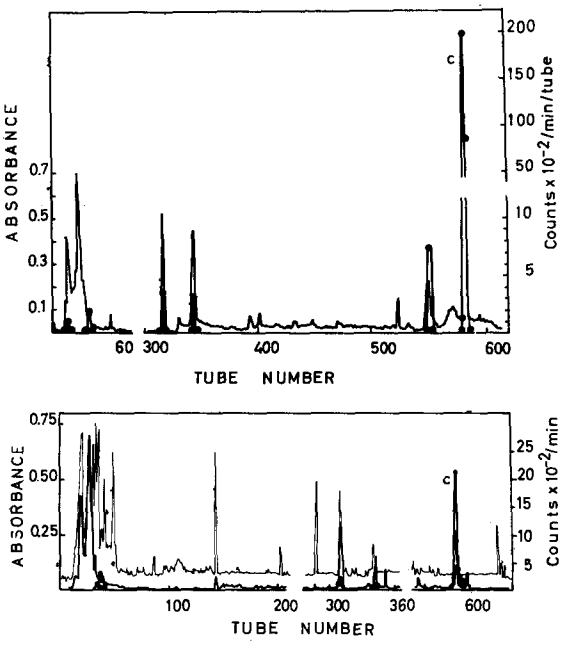

Fig. 3. Chromatography on a Dowex 1-X2 column (1 cm × 150 cm) of the tryptic digest of a 68-mg sample of $^{14}CO_2$–ribulosediphosphate carboxylase complex stabilized by methylation with diazomethane. (a) Undiffusible tryptic peptides. Buffers changed at 105, 245, 310, 362, 400, 435, 521 and 555 fractions, respectively. - - - - -, absorbance at 500 mμ (Folin); ●—●, ^{14}C radioactivity per fraction. (b) Diffusible tryptic peptides. Buffers changed at 75, 240, 310, 362, 413, 448, 523 and 557 fractions respectively. Dark line, absorbance at 500 mμ (Folin); light line, absorbance at 570 mμ (ninhydrin); ●- - -●, ^{14}C radioactivity per fraction. Other conditions as in Fig. 1.

Fig. 4.(a) Absorption spectrum of radioactive peptide c after pepsin hydrolysis and chromatography in 2 M acetic acid on a Dowex 1-X2 column. (b) Comparison of the spectral changes of the radioactive peptide c after pepsin hydrolysis as affected by pH. A, in 0.06 M NaOH: B, in 0.08 M HCl.

Previous studies had shown that zinc ions form a complex with the enzyme and inhibit the carboxylation of ribulose 1,5-diphosphate[11]. Since it was of interest to see whether the inhibition is due to binding of Zn^{2+} on the active site of the enzyme on which CO_2 is bound, before incubation with $^{14}CO_2$, the enzyme was treated with Zn^{2+}. The peptide elution pattern showed that the ^{14}C-labeled peptide was eluted in the same fraction, but its relative radioactivity was reduced to one-fifth of the radioactivity of the peptide formed upon digestion of the $^{14}CO_2$–enzyme complex, which was prepared with native enzyme (see Figs. 2 and 3).

The chromatographic and radioactivity pattern of the tryptic digest of ribulose-diphosphate carboxylase, separated from spinach leaves grown in [4-^{14}C] δ-aminolevulinic acid, showed that, from all the peptides eluted, only peptides c, a and b were radioactive, the last two but slightly. This observation shows that δ-aminolevulenic acid is a precursor of the chromophore part of the enzyme.

DISCUSSION

The observation, that from all the peptides formed after tryptic digestion only one is highly radioactive, suggests that the CO_2 binding on the enzyme is highly specific, as one would expect for a binding to the active site. If unspecific binding due to the formation of carbamates occurred[6], a distribution of the radioactivity to all peptides formed would be expected. Moreover, the observation that the zinc ions, an inhibitor of the enzyme, suppresses the CO_2–enzyme formation reaction is another

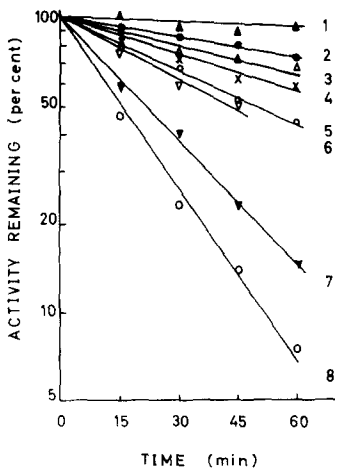

Fig. 5. Time curve for the inhibition of ribulosediphosphate carboxylase by iodoacetamide at 25° in 0.1 M Tris buffer (pH 8.0) in the presence of different protectors. All reaction mixtures contained: enzyme, 0.9 mg/ml; iodoacetamide, 0.5 mM; protector (1), carbamyl phosphate, 0.5 mM; (2) fructose 1-phosphate, 1.5 mM; (3) ribulose 1,5-diphosphate, 0.5 mM; (4) Na_2SO_4, 15 mM; (5) fructose 1,6-diphosphate, 1.0 mM; (6) Na_2HPO_4, 15 mM; (7) fructose 1,6-diphosphate, 0.1 mM; (8) control, no protector. The iodoacetamide was added at zero time to the reaction mixture containing the enzyme, Tris buffer and protector, and the reaction was allowed to proceed at 25°. Aliquots (50 μl) were removed at predetermined intervals of time and added to tubes precooled at 0°, containing 50 μl of 0.01 M cysteine in 0.2 M Tris buffer (pH 8.0). The other components of the assay were then added, and the reaction mixture was incubated at 25° for 10 min.

proof of the specificity of CO_2 attachment, and it shows unquestionably that the CO_2–enzyme complex is an intermediate in the dismutation reaction.

Earlier work[12] had shown that ribulosediphosphate carboxylase is inhibited by *p*-chloromercuribenzoate and iodoacetamide, thus implicating an –SH group in the active site of the enzyme. Lately RABIN AND TROWN[2] reported that ribulose 1,5-diphosphate, one of the substrates, protects the enzyme against iodoacetamide inhibition. On the basis of this protection it was concluded that the suppression of the inhibition is due to hemimercaptole formation between the substrate and the enzyme at the same –SH group where the alkylation by iodoacetamide occurs, the hemimercaptol formed being considered as an intermediate of the enzymatic reaction. However, it has been found in our laboratory[3] that other sugar phosphates, namely fructose 1,6-diphosphate, fructose 1-phosphate, fructose 6-phosphate, as well as carbamyl phosphate and phosphate and sulfate ions, also protect the enzyme against iodoacetamide inhibition (see Fig. 5). If these compounds were in fact protecting the enzyme by reaction at the active site, thereby preventing the alkylation from taking place, one would also expect them to be competitive inhibitors of the enzyme. However, all efforts to establish such an inhibition of the enzymatic activity by these protectors have failed. In view of our results therefore, we cannot decide whether the hemimercaptol formation between ribulose 1,5-diphosphate and the –SH group of the enzyme is in fact an intermediate of the enzymatic reaction.

A possibility of the existence of amino acid residues on the protein responsible for keeping the enzyme structure in the right spatial configuration, –SH groups for example, which can be alkylated by iodoacetamide with concomitant change in structure and inactivation, cannot therefore be disregarded. The protector may thus be thought to be bound on the same structure determining the site or sites *via* hydrogen bonds not allowing attack by iodoacetamide and keeping the enzyme in the active form. This site, however, cannot be considered as the active site of the enzyme since the latter is highly specific for the substrate.

The findings that one peptide of the tryptic digested enzyme was distinctly yellow, that this same peptide was highly radioactive when it was separated from the complex stabilized by diazomethane $^{14}CO_2$–enzyme, and that the radioactivity was proportional to the intensity of the yellow color, are of great interest. The identity of Fraction I protein, protochlorophyll holochrome and ribulosediphosphate carboxylase has often been speculated upon the last few years[13,14]. However, all the studies have been based on sedimentation rates, diffusion behavior or molecular weight and solubility of the proteins, which cannot unequivocally prove the conclusions reached. One of the anomalies was that no pigment was reported to be present in ribulosediphosphate carboxylase. In our experiments with this enzyme we have always noticed the preparations to be slightly yellow no matter how pure they were. Moreover, we have always noticed that upon elution of the enzyme from CM-Sephadex columns, the color accompanied the enzyme band. However, since the enzyme has not been purified sufficiently to decide whether the color is due to a contaminant, no attempts to characterize the pigment have been made. The question as to the nature and function of the chromophore is now reopened, since the pigment is part of the enzyme and close to the active site of CO_2 attachment. The absorption spectrum characteristics of the colored peptide cannot show whether or not the chromophore is a chlorophyllide, since it becomes modified during the series of treatments for the isolation of the ^{14}C-labeled

peptide. However, the results of some preliminary experiments designed to show whether the δ-aminolevulinic acid is a precursor of the colored part of the peptide were very promising. After separation of the tryptic peptides of ribulosediphosphate carboxylase, isolated from spinach leaves stood in [4-^{14}C]δ-aminolevulinic acid, radioactivity was recovered only in the colored peptides. This is positive evidence for the chromophore being a pyrolle-containing compound. Definite proof, however, must await further experiments. Therefore, no firm conclusion can be drawn yet as to the nature and function of the chromophore in ribulosediphosphate carboxylase. Moreover, it is not known if it has anything to do with the action of the enzyme, or if the $^{14}CO_2$ is bound to the colored moiety of the peptide or to the side chain of an amino acid.

It has been a common finding that there is a difference in the carboxylation reactions catalyzed by ribulosediphosphate carboxylase *in vivo* and *in vitro*. The reaction *in vitro* is a non-reductive carboxylation of ribulose 1,5-diphosphate leading to the formation of two molecules of 3-phospho-D-glycerate; the reaction *in vivo*, however, (based on kinetic evidence with whole cells), may possibly proceed as a reductive carboxylation reaction leading to the formation of one molecule of 3-phospho-D-glycerate and one molecule of phosphoglyceraldehyde[15]. This difference may be due to some modefication modified during isolation and purification procedures, the modification destroying the chromophore. It may thus be thought that structurally intact ribulosediphosphate carboxylase with the chromophore part not disrupted—as in the live cell—is capable of catalyzing the reductive carboxylation reaction whereas the same enzyme after the series of isolation steps is not able to do so.

Attempts to characterize the chromophore, the nature of its action and binding on the enzyme, and to elucidate the mechanism of ribulosediphosphate carboxylase catalysis are under way in our laboratory. The isolation of a small fragment of the protein containing the active site renders the elucidation of the enzymatic catalysis easier.

ACKNOWLEDGEMENT

This investigation was supported in part by a Charles F. Kettering Foundation research grant.

REFERENCES

1 G. AKOYUNOGLOU AND M. CALVIN, *Biochem. Z.*, 338 (1963) 20.
2 B. R. RABIN AND P. W. TROWN, *Nature*, 202 (1964) 1290.
3 J. H. ARGYROUDI-AKOYUNOGLOU AND G. AKOYUNOGLOU, Nature, 213 (1967) 287.
4 G. AKOYUNOGLOU, Ph. D. Thesis, *University of California Lawrence Radiation Laboratory Report*, UCRL-10352 (1962).
5 N. G. PON, Ph. D. Thesis, *University of California Lawrence Radiation Laboratory Report*, UCRL-9373 (1960).
6 G. AKOYUNOGLOU AND M. CALVIN, *J. Org. Chem.*, 28 (1963) 1484.
7 G. FUNATSU, *Biochemistry*, 3 (1964) 1351.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
10 H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 2, Interscience Publishers, New York, 1961, p. 406.

11 G. AKOYUNOGLOU AND J. H. ARGYROUDI-AKOYUNOGLOU, submitted for publication.
12 J. MAYAUDON, A. A. BENSON AND M. CALVIN, *Biochim. Biophys. Acta*, 23 (1957) 342.
13 D. W. KUPKE AND C. S. FRENCH, in W. RUHLAND, *Encyclopedia of Plant Physiology*, Vol. 5, Part 1, Springer, Berlin, 1960, p. 318.
14 P. W. TROWN, *Biochemistry*, 4 (1965) 908.
15 J. A. BASSHAM, *Ann. Rev. Plant Physiol.*, 15 (1964) 101.