ON THE PROTECTION OF RIBULOSE DIPHOSPHATE CARBOXYLASE FROM IODOACETAMIDE INHIBITION

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The enzyme ribulose diphosphate carboxylase (RuDP-carboxylase),known to be inactivated by iodoacetamide (IAM), (Mayaudon, Benson and Calvin, 1957), has been found to be protected by RuDP, one of the substrates (Rabin and Trown, 1964), as well as by carbamyl phosphates (CBMP) sulphate and phosphate ions, and different sugar phosphates (Argyroudi-Akoyunoglou and Akoyunoglou, 1967). Since these protectors were not found to be competitive inhibitors of the enzyme the mechanism of their protection has been suggested to involve binding of the protectors to sites on the enzyme not related to the active site.However, these sites have been considered to be responsible for keeping the enzyme in its native tertiary structure which is destroyed by IAM alkylation rendering thus the enzyme inactive.(Argyroudi-Akoyunoglou and Akoyunoglou, 1967).

The use of coenzymes, substrates or competitive inhibitors for protection of enzymes from inactivation due to alkylation by IAM has been described in several cases. (Watts and Rabin, 1962; Walsh et al., 1962;Szewczuk and Connel, 1965; Li and Vallee, 1963; Virden and Watts, 1966).Most enzymes inhibited by IAM have been considered to form carboxymethylcysteine, and in some cases ^{14}C-labelled carboxymethylcysteine has been isolated (Rabin and Trown, 1964; Grazietal, 1965; Light, 1964). It has been very clearly demonstrated, however, that the loss of enzymatic activity of ribonuclease by IAM alkylation is due to the formation of carboxymethlhistidine, and moreover, that phosphate ions can protect the enzyme by binding at those sites (Crestfield, et al., 1963a; 1963b). The imidazole group of the histidyl residue at the active site of trypsin has been found also to be alkylated by IAM (Inagami, 1965). Mention should be made of the recent finding that glycolic acid was liberated from samples of alkylated by IAM gamma-

glutamyl transpeptidase in addition to carboxymethlhistidine, carboxymethylcysteine and carboxymethyllysine (Szewezuk and Connell, 1965). ^{14}C-labelled carboxymethylcysteine has been isolated from alkylated RuDP-carboxylase in addition to some unidentified radioactive products (Rabin and Trown, 1964).

In order to get more information as to the protection of RuDP-carboxylase by CBMP against IAM inhibition and find out by which mechanism IAM acts as far as this enzyme is concerned we undertook this study.

The results of this communication clearly show that the first amino-acid residue to be alkylated in the protein by IAM is cysteine. However, the protective effect of CBMP against IAM inhibition has nothing to do with CBMP reaction on the site of IAM alkylation.

Materials and methods

The enzyme RuDP-carboxylase was prepared from spinach chloroplasts, Spinacea Oleracea, as previously described (Akoyunoglou et al., 1967).The enzymatic activity was measured by determining the 3-phosphoglyceric acid formed as acid stable radioactivity after incubation of the enzyme with $MgCl_2$, RuDP and $HC^{14}O_3^-$. The protein concentration was estimated spectrophotometrically using the relationship OD_{280} X 0.6 = mg protein/ml.(Akoyunoglou et al., 1967). The C^{14}-IAM was a product of New England Nuclear Co., Boston, Mass. The CBMP as the dilithium salt, was a product of Nutritional Biochemicals Co., Cleveland, Ohio. Three sets of experiments were performed : in the first the enzyme was incubated with C^{14}-IAM in the presence or absence of CBMP then dialyzed, lyophylized, and the lyophylized powder was hydrolyzed.Another enzyme sample also was allowed to react with cold IAM in the presence of CBMP for one hour, it was then dialyzed and into the dialyzed enzyme mixture new C^{14}-IAM was added. Reaction took place for one more hour and then new dialysis was done. The dialyzed alkylated protein was then lyophylized and hydrolyzed.

In the second set of experiments the enzyme was allowed to react with C^{14} -IAM in the presence or absence of CBMP and at different intervals of time aliquots of the reaction mixtures were removed, immediately dialyzed at 0° C, lyophylized and then hydrolyzed.

In the third set of experiments the enzyme was allowed to react with cold IAM in the presecne or absence of CBMP for 24 hours. After that it was dialyzed and then incubated with new C^{14}-IAM for 38 more hours. Then it was dialyzed, lyophylized and hydrolyzed.

The completeness of dialysis was checked by counting the dialysate

and following the unreacted C^{14}-IAM loss.

The hydrolysis of the enzyme samples took place in 5.7 N HCL for 22 hrs at 118° C in vacuo. The resulting amino acids were separated according to Moore, Spackman and Stein (1958) on columns of Aminex-MS (obtained from Bio-Rad Laboratories, Richmond, Calif.) 1 x 74 cm.The C^{14}-radioactivity was counted in a thin window gas flow counter. The ninhydrin of the separated amino acids was done according to Moore and Stein (1954).

Results and Discussion

Carbamyl phosphate is a very potent protector of the enzymatic activity of RuDP-carboxylase from its inhibition due to IAM (Argyroudi-Akoyunoglou and Akoyunoglou, 1967). There were two alternatives as to the mechanism of this protection : either that the protection is due to binding on the alkylated by IAM site or sites or that the protection is one of structure determining character, i.e. it is due to binding on certain amino acids on the enzyme responsible for the configuration of the native protein. For the first alternative it was also concluded earlier that this site or sites could not be "the active" site because that would be highly specific for the substrate.

Since it was not known if the protector protects all the alkylated by IAM residues on the protein or just some of them we performed some preliminary experiments in which enzyme reacted with IAM in the presence of CBMP for one hour was dialyzed and then incubated with new IAM. In both steps the enzymatic activity was measured and it was found that although the enzyme after dialysis is fully active, it is as well inhibited by the new addition of IAM. This finding was suggestive of a mechanism by which the CBMP protects certain alkylated sites by not allowing IAM to react. We therefore thought to use C^{14}-IAM in our experiments to follow closer the alkylation reaction.

The representative results shown in fig.1 and table I indicate clearly that alkylation of the enzyme by IAM results in the formation of C^{14}-carboxymethylcysteine(fist peak of radioactivity) and 1 or 3-C^{14}-carboxymethylhistidine (second peak of radioactivity). The identification is based on the elution volume from the same column of control alkylated by IAM cysteine and histidine. However, as it is evident from table I the presence of carbamyl phosphate in the incubation mixture does not affect the C^{14}-binding, i.e. the alkylation by IAM, since the same bound radioactivity is recovered, both in the presence and absence of CBMP. It can be calculated that in one hour 1.76 μmoles of carboxymethylcysteine and 0.15 μmoles

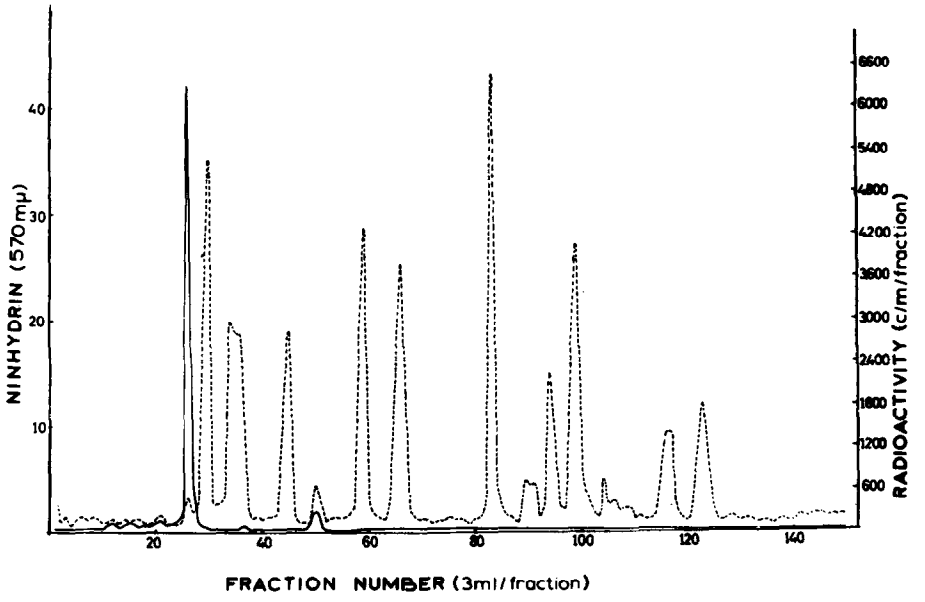

Fig.1. 4.59 mg RuDP-carboxylase were incubated with C^{14}-IAM at a final concentration of 5 mM in 0.1 M Tris buffer pH 8.0 at 25° for one hour. Final volume one ml. The mixture was then dialyzed for 24 hours at 2° C versus 0.01 M Tris buffer pH 8.0, lyophylized and hydrolyzed. The hydrolysate was lyophylized and the amino acids eluted from a column of Aminex-MS 1 x 74 cm according to Moore, Spackman and Stein (1958). Ninhydrin was done by the method of Moore and Stein (1954) on 2.5 ml aliquots of each fraction, ———, C^{14}- radioactivity. ---------, absorbance at 570 mμ.

Table I

Radioactivity recovered as carboxymethylcysteine and carboxymethylhistidine after reaction of RuDP-carboxylase with C^{14}-IAM in the presence and absence of CBMP.

Reaction conditions	Carboxymethyl-cystein c/m	carboxymethyl-histidine c/m
E + C^{14}-IAM	7092	600
E + C^{14}-IAM + CBMP	7188	522
E + IAM + CBMP $\xrightarrow{\text{dialysis}}$ dialyzed sample + C^{14}-IAM	5880	1398

4.59 mg enzyme were incubated with C^{14}-IAM at a final concentration of 5 mM (1.2 μc/μmole) in 0.1 M Tris buffer pH 8.0 at 25° for one hour in the presence and absence of 10 mM CBMP. Final volume 1 ml. The mixture was then dialyzed for 24 hours at 2° versus 0.01 M Tris buffer, pH 8.0, lyophylized, hydrolyzed, and the amino acid residues separated. In the third experiment we had the same conditions as above except that cold IAM was used and after dialysis C^{14}-IAM was added in a final concentration of 5 mM (1.2 μc/mole). After 1 more hour of reaction at 25° new dialysis took place followed by lyophilization and hydrolysis.

of carboxymethylhistidine are formed per μmole enzyme, based on a molecular wieght of about 550,000. Moreover new addition of C^{14}-IAM to an enzyme sample already alkylated by cold IAM in the presence of CBMP, results as well in C^{14}-binding (table I).

Since both cysteine and histidine are alkylated by IAM after one hour reaction, fig.1, and in an effort to find out which one of the two amino-acids is responsible for loss of enzymatic activity we performed some experiments in which the rate of the alkylated amino acid residues formation was studied in the presence or absence of CBMP. The results are shown in Table II. It is clear that as the time of incubation of the enzyme with C^{14}-IAM increases the amount of C^{14}-carboxymethylated derivatives is also increased.

Carboxymethylcysteine is present even after 5 min.of reaction, but only after 30 min. the formation of C^{14}-carboxymethylhistidine is evident, while C^{14}-carboxymethyl-cysteine is the sole product up to that time. However, reaction with C^{14}-IAM takes place even in the presence of CBMP as evidenced by the C^{14}-carboxymethylated derivatives isolated.

Table II

Rate of alkylation of RuDP-carboxylase by C^{14}-IAM in the presence and absence of CBMP.

Reaction Conditions	Reaction Time (min)	CM-Cys c/m	CM-His c/m	CM-Cys μmoles (x 10^3)	μmoles CM-Cys / μmole Enzyme
E + IAM	5	4330		4.5	0.75
E + IAM + CBMP	5	4000		4.17	0.70
E + IAM	15	6360		6.62	1.1
E + IAM + CBMP	15	5780		6.00	1.00
E + IAM	30	7960	158	8.3	1.38
E + IAM + CBMP	30	6800	83	7.1	1.18
E + IAM	60	9860	312	10.3	1.72
E + IAM + CBMP	60	7845	106	8.15	1.36

17 mg enzyme were allowed to react with C^{14}-IAM at a final concentration of 5 mM (2.4μc/μmole) in 0.1 M Tris buffer, pH 8.0, at 25°C in the presence or absence of CBMP, at a concentration of 20 mM.Final volume 2.5 mls. Aliquots of 0.5 mls were withdrawn at different intervals of time, and dialyzed at 2° C for 5 days versus 0.01 M Tris buffer pH 8.0. The dialyzed samples were then lyophylized hydrolyzed and analyzed as described in the text.

Since the IAM binding is continuously increased even after one hour incubation, time at which the enzymatic activity left is negligible in the absence of CBMP, we conducted another type of experiment in which the effect of a longer time of incubation of the enzyme with IAM was studied. The enzyme was incubated with cold IAM in the presence and absence of CBMP for 24 hours.The enzymatic activity was measured in both samples and then they were dialyzed.After dialysis the samples were reacted again with a new quantity of C^{14}-IAM for 38 more hours.As it is clearly shown in table III the CBMP is able to protect the enzyme against IAM inhibition for at least the 24-hour period tested.

This new addition of C^{14}-IAM to already alkylated enzyme by cold IAM for 24 hours, results in formation of C^{14}-carboxymethylcysteine, C^{14}-carboxymethylhistidine and other carboxymethylated amino acid residues (Fig. 2). This finding shows that the alkylation process proceeds very slowly under these conditions. It also shows that the 24-hour incubation of the

T a b l e III

Specific activity of enzyme after 24-hour reaction with IAM in the presence and absence of CBMP

Sample	c/m/mg protein
Control (E without IAM)	2,200,000
E+IAM	4,700
E+CBMP+IAM	1,460,000

4 mg enzyme in 0.01 M Tris, pH 8.0, were allowed to react with IAM at a final concentration of 5 mM at 25° (for 24 hours. Final volume 1 ml.CBMP was added solid to have a final concentration of 20 ml.After reaction, aliquots containing 40 µg enzyme were removed and added into pre-cooled at 0° C tubes containing (µmoles); Tris buffer (pH 8.0), 20; Mg^{++}, 2.5; Cysteine, 4; then $HC^{14}O_3^-$ (specific activity 5.5 µc/µmole), 1.5, and RuDP, 0.11 were added.Final volume 0.2 ml.Incubation of the reaction mixture, 10 min.at 25° C.

enzyme with cold IAM is not enough for complete alkylation of all the groups on the protein even though there is an excess of IAM.

It is interesting to note that the same amount of C^{14}-carboxymethyl derivatives are formed during the 38-hour period in both cases.i.e. both the fully active enzyme sample in the presence of the protector and the inactivated by IAM one are carboxymethylated to the same extent. It can be calculated that about 6 µmoles of carboxymethylcysteine and 1 µmole

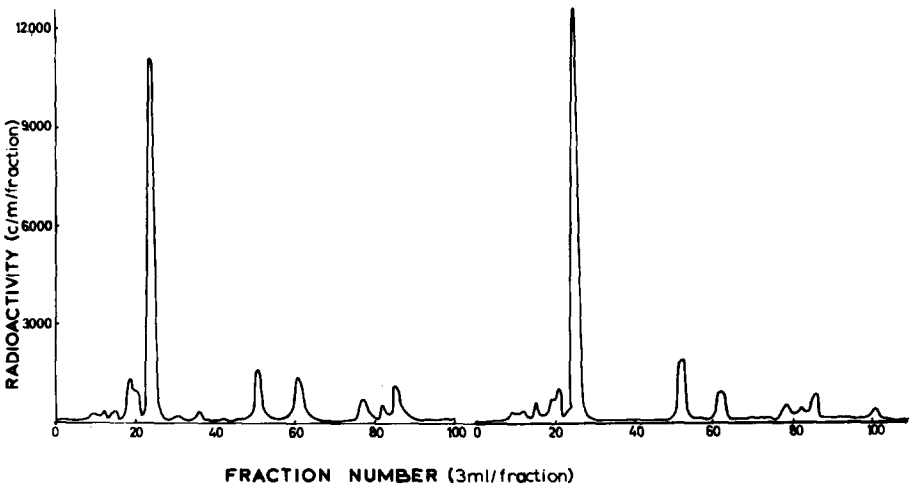

Fig.2 . Separation of the carboxymethylated amino acids from alkylated by cold IAM RuDP-carboxylase incubated with new C^{14}-IAM. 4.05 mg enzyme were incubated with IAM, final concentration 5 mM, at 25° C in 0.01 M Tris buffer pH 8.0 for 24 hours, in the presence or absence of CBMP (20 mM). Final volume 1 ml. Then dialysis took place versus Tris buffer and after that C^{14}-IAM was added (1.2 μc/μmole, 5 mM final). Incubation took place at 25° C for 38 more hours and then the samples were dialyzed, lyophylized and hydrolyzed. The separation of the amino acid residues took place as described in the text.
Left : Incubation in the absence of CBMP.
Right: Incubation in the presence of CBMP.

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of carboxymethylhistidine are formed per μmole of enzyme in both cases.

All these findings show clearly that the protection of the enzyme by CBMP is not due to binding of the protector on the active site which is alkylated by IAM. Moreover the inhibition of the enzyme by IAM is not due to the alkylation reaction since the same amount of carboxymethylated derivatives are formed when the enzyme is alkylated in the presence of CBMP in which case the enzymatic activity is not lost.

It would be very interesting to show that a completely alkylated enzyme by IAM in the presence of CBMP is still fully active. Our efforts to do such an experiment were not successfull because the alkylation reaction proceeds very slowly at these conditions and longer incubation of the enzyme at 25° C results in inactivation of the enzyme even in the absence of the alkylating agent due to denaturation. However, experiments are under way to find the more favorable conditions for such a study.

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