Glutamine Active Site of Formylglycinamide Ribonucleotide Amidotransferase. 1. Labeling of the Enzyme with Iodoacetate†

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ABSTRACT: A two-step method for labeling the glutamine active site of formylglycinamide ribonucleotide (FGAR) amidotransferase from chicken liver has been developed in which reaction of all other reactive groups with unlabeled iodoacetate is followed by specific labeling of the glutamine site with radioactive reagent. A study of the reaction as a function of duration, temperature, and pH of the incubation as well as concentration of iodoacetate has revealed that two nonessential groups of the enzyme react in the presence of glutamine and that this modified enzyme is relatively resistant to further carboxymethylation. When this modified enzyme was incubated with radioactive iodoacetate in the presence of FGAR, ATP, and Mg^{2+} after removal of glutamine by dialysis, about 1 mol of radioactive iodoacetate was incorporated per mol of enzyme with inactivation. This method permits labeling of the active site for glutamine without the use of glutamine analogues.

Formylglycinamide ribonucleotide amidotransferase (2-formamido-*N*-ribosylacetamide-5′-phosphate:L-glutamine amido-ligase (adenosine diphosphate) EC 6.3.5.3) catalyzes the reaction in which the amide nitrogen of glutamine is transferred to formylglycinamide ribonucleotide (FGAR)[1] to form formylglycinamidine ribonucleotide (FGAM) by coupling the energy of ATP splitting to the formation of the new carbon–nitrogen bond (reaction 1):

$$\text{FGAR} + \text{L-glutamine} + \text{ATP} + H_2O \rightarrow \text{FGAM} + \text{L-glutamate} + \text{ADP} + P_i \quad (1)$$

FGAR amidotransferase is composed of a single multifunctional polypeptide of 133 000 daltons, and can, in the absence of other substrates, form a relatively stable glutamine–enzyme complex, which gradually decomposes to glutamate and enzyme with a half-life of 125 min at 4 °C (Mizobuchi and Buchanan, 1968b).

Investigation of the glutamine reactive site of the bacterial enzyme has been made possible by use of ^{14}C-labeled L-azaserine, an analogue of glutamine and the first active-site-directed reagent to be employed for this purpose (Dawid et al., 1963). Azaserine causes the irreversible inactivation of the enzyme by covalent reaction with a specific cysteinyl sulfhydryl group. A pentapeptide sequence at the location of this sulfhydryl group was identified as alanylleucylglycylvalylcysteine. Further experiments with the chicken liver enzyme have also shown that iodoacetate and azaserine react at the same site in the enzyme, i.e., the glutamine reactive site (Schroeder et al., 1969; Mizobuchi et al., 1968). Therefore, it should be possible to label the glutamine active site selectively with [^{14}C]iodoacetate, if other reactive groups are first covered or masked before incubation with the radioactive inhibitor. The purpose of the present paper has been to establish the basic conditions for the labeling of this azaserine-reactive site so that a relatively long radioactive peptide could be isolated in sufficient quantity for sequence analysis. The amino acid composition and partial sequence of the labeled peptide are reported in the accompanying paper (Ohnoki et al., 1977).

Experimental Procedure

Materials

The ammonium salt of FGAR was enzymatically prepared by the method of Hartman et al. (1956) with the modification that pigeon liver extract was replaced by an equivalent activity from chicken liver. Conversion of the ammonium salt of FGAR to the potassium salt was achieved by column chromatography on Dowex 50W-X12 (K^+). L-Glutamine was purchased from Sigma Chemical Company. Iodoacetate, a product of Eastman Kodak, was recrystallized twice from chloroform. [1-^{14}C]-Iodoacetate was obtained from New England Nuclear. In order to remove free iodine from a very small sample of radioactive material, it was dissolved in chloroform, dried gently under a nitrogen stream at 50 °C for a few minutes, and again dissolved in chloroform, and the above procedure was repeated several times. After the third or fourth such treatment a completely colorless powder freed from iodine was obtained. The yield was 70–80%.

5-Aminoimidazole ribonucleotide (AIR) synthetase was the ammonium sulfate fraction of pigeon liver described previously (Levenberg and Buchanan, 1957). This fraction was dialyzed briefly against 0.1 M potassium phosphate buffer (pH 7.4) and lyophilized to dryness.

Methods

Enzyme Purification. The published procedure (Mizobuchi and Buchanan, 1968a) to prepare FGAR amidotransferase from chicken liver was used with several modifications. The use of calcium phosphate gel in step V was replaced by gel filtration with Sephadex G-200. A linear gradient system from 2 mM to 0.1 M potassium phosphate buffer (pH 7.2) was used in column chromatography with DEAE-cellulose in steps VI and VII. In order to increase the column capacity, calcium phosphate gel in the last step (step VIII) was packed without mixing with cellulose powder. A reasonable flow rate was obtained without use of the latter. Another modification of the previous method (Mizobuchi and Buchanan, 1968a) of enzyme purification was that all buffers were standardized to contain

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[1] Abbreviations used are: FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, aminoimidazole ribonucleotide.

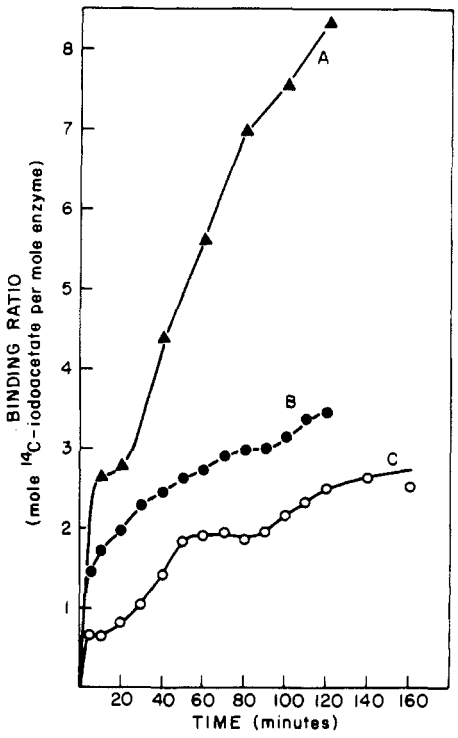

FIGURE 1: Time course of iodoacetate binding to the enzyme in the presence of glutamine: (A, ▲) FGAR amidotransferase (2.13 nmol) was incubated with glutamine (100 mM), ATP (5 mM), $MgCl_2$ (5 mM), [^{14}C]iodoacetate (24 mM), and potassium phosphate (pH 7.7) (100 mM), in a total volume of 0.2 mL at 37 °C; (B, ●, and C, ○) FGAR amidotransferase (8.5 nmol) was incubated with glutamine (100 mM) and [^{14}C]iodoacetate (24 mM) and either glycylglycine (pH 8.0) (200 mM) or potassium phosphate (pH 6.5) (200 mM), respectively, in a total volume of 1 mL at 25 °C. Aliquots were removed and assayed for enzyme activity and binding of iodoacetate at the times indicated.

1 mM glutamine, 10% glycerol, 0.1 mM dithiothreitol, and 1 mM EDTA but with omission of $MgCl_2$. The concentration of the enzyme was calculated by use of 186 000 M^{-1} cm^{-1} as the molecular extinction coefficient at 280 nm or 6.65×10^{10} units per mol of the enzyme under the conditions of the standard assay procedure for activity.

Assay of Enzyme Activity. FGAR amidotransferase activity was measured by the standard assay procedure described in detail in a previous paper (Mizobuchi and Buchanan, 1968a). A solution of the coupling enzyme, AIR synthetase, was always freshly prepared in a concentration of 10 mg/mL.

Progressive Inactivation Studies. Experiments were typically performed by incubating enzyme with iodoacetate and substrates in a small volume (0.1 to 0.2 mL) for various times. When the experiment was performed in the presence of glutamine, the enzyme was incubated with glutamine at 37 °C for 5 min before addition of iodoacetate. Aliquots (2–10 μL) were then removed for standard assay with glutamine as nitrogen source (Mizobuchi and Buchanan, 1968a). When the concentration of enzyme in the incubation mixture was too high to assay directly, an aliquot was diluted 20- to 50-fold with chilled 0.1 M potassium phosphate buffer (pH 6.5–7.0) and then a suitable amount was used. The half-time required for 50% inhibition was estimated from the line describing the time course of inhibition. When the inhibition rate was slow, $t_{1/2}$ was calculated from eq 2:

$$t_{1/2} = t \log 2/(2 - \log x) \quad (2)$$

where x is the remaining relative activity (percent) and t is the time of incubation. In all instances the course of inhibition followed first-order kinetics.

Assay of Binding Ratio. Enzyme was incubated with radioactive iodoacetate under various conditions in the same way as described above, and aliquots (50–100 μL) were removed for assay of bound radioactivity by precipitation of the protein in 5% trichloroacetic acid. The binding ratio of iodoacetate to enzyme under the conditions described for reaction of nonessential groups was calculated on the basis of protein concentration determined by absorbance at 280 nm. The binding of radioactive iodoacetate to the masked enzyme in the specific labeling step was measured on the basis of the concentration of active enzyme as determined by enzymatic activity. In order to determine the amount of radioactive material absorbed to the filter paper but not bound to enzyme, the same amount of reaction mixture without [^{14}C]iodoacetate was first treated with 5% trichloroacetic acid, and then [^{14}C]iodoacetate was added. The binding ratios were corrected for this blank value. The term "binding" of iodoacetate is used to designate the covalent attachment of the carboxymethyl moiety to the enzyme with displacement of the iodine.

Results

The purpose of the following experiments has been to determine the conditions for labeling the amino acid residue at the glutamine-reactive site of FGAR amidotransferase with [^{14}C]iodoacetate after controlled reaction of enzymatically nonessential groups with unlabeled reagent. The exact identification of these nonessential groups has not been important, since our main objective has been to reduce to a negligible quantity the reaction of these residues with radioactive iodoacetate.

Conditions for Reaction of Nonessential Groups in the Presence of Glutamine

Time Course of Iodoacetate Binding. Previous investigations (Schroeder et al., 1969) have shown that in the presence of glutamine FGAR amidotransferase can be protected from inactivation by iodoacetate, although residues nonessential for enzyme activity do react with this reagent. To determine the incubation time that is required for carboxymethylation of the reactive groups other than the one at the glutamine active site, the binding of iodoacetate during a relatively long incubation was examined. When the enzyme was incubated with 24 mM iodoacetate at 37 °C, the binding of iodoacetate gradually increased over the period of 2 h and did not stop at a given level for a sufficient period of time to control the reaction (Figure 1, curve A). A slight inactivation of the enzyme (approximately 10%) was observed at 2 h. When the enzyme was incubated with the same concentration of iodoacetate at 25 °C, carboxymethylation of the enzyme leveled off after 80 to 90 min at pH 8.0 (Figure 1, curve B). At pH 6.5 there was a halt in the reaction at 10 min and a longer arrest after 50 to 90 min (Figure 1, curve C), although the reaction with iodoacetate started again at 90 min in both cases. Enzyme activity was fully maintained at 25 °C for over 2 h at both pH values. These results show that iodoacetate reacts with some nonessential groups of the enzyme more specifically at 25 °C than at 38 °C and at pH 6.5 rather than at pH 8.

Since FGAR amidotransferase contains approximately 18 free sulfhydryl groups when completely denatured (Schroeder et al., 1969), it was necessary to titrate with unlabeled reagent the rapidly reacting residues under conditions that did not lead to further exposure of other groups during specific labeling of

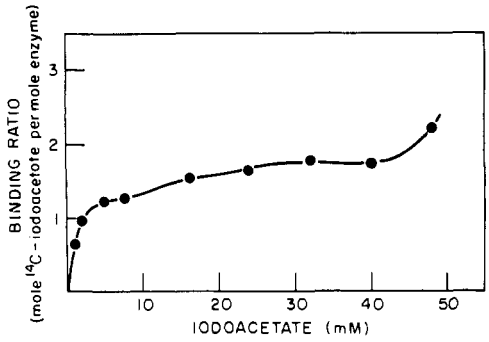

FIGURE 2: Effect of concentration of iodoacetate on its binding to enzyme in the presence of glutamine. FGAR amidotransferase (0.43 nmol) was incubated with glutamine (100 mM), ATP (5 mM), $MgCl_2$ (5 mM), and potassium phosphate (pH 7.2) (100 mM), in a volume of 0.1 mL containing the indicated amount of iodoacetate at 25 °C for 60 min. Aliquots (2 μL) were removed for enzyme activity assay (not recorded) and the rest was used for the binding assay (●).

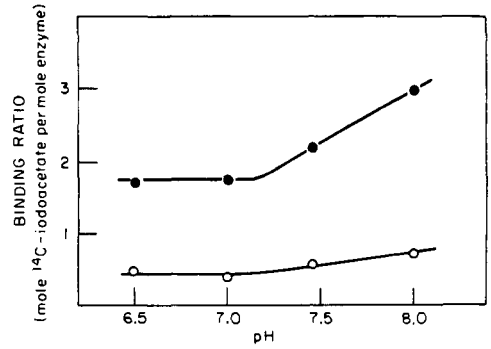

FIGURE 3: pH dependency of iodoacetate binding in the presence of glutamine. FGAR amidotransferase (0.43 nmol) was incubated with glutamine (100 mM), buffer (200 mM) of pH indicated, and [^{14}C]iodoacetate (○, 0.4 mM; ●, 32 mM) in a volume of 0.1 mL at 25 °C for 60 min. The buffers used are potassium phosphate (pH 6.5–7.5) or glycylglycine (pH 8.0). Aliquots (2 μL) were removed for activity assay and the rest was used for binding assay.

the residue at the glutamine site. Therefore, the reaction conditions were explored in more detail at 25 °C.

Concentration Effect of Iodoacetate. Figure 2 shows the binding ratio of iodoacetate to enzyme when the enzyme was incubated with ATP and Mg^{2+} in the presence of glutamine at various concentrations of iodoacetate for 60 min at 25 °C. Two plateaus of binding were observed. The first one appears at 5 mM and the second between an iodoacetate concentration of about 20 and 40 mM. The same result was obtained when incubation was carried out with only glutamine (100 mM) as substrate. In the latter case, activity was fully maintained during incubation with up to 48 mM iodoacetate, and only slight inhibition (10–15%) was observed at the concentration of 40–50 mM iodoacetate when ATP and Mg^{2+} were present in addition to glutamine. From consideration of graphs illustrating the time course of reaction (Figure 1) and the effect of iodoacetate concentration (Figure 2), we conclude that the enzyme has two reactive residues other than the one involved in glutamine binding. There seems to be considerable difference in the reactivities of these two nonessential groups at 25 °C, the first being titrated at a relatively low concentration of iodoacetate and the second over a much higher concentration of reagent. The carboxymethylation of a third group is only observed at a very high concentration of iodoacetate.

pH Dependency of Iodoacetate Binding. Although the foregoing experiments had indicated a more limited and controlled reaction of nonessential groups at lower pH values, we have undertaken a more complete study of the reactivity of these residues as a function of pH and at two concentrations of iodoacetate.

The same amount of glutamine–enzyme complex as used in the experiment reported in Figure 2 was incubated with radioactive iodoacetate at 25 °C in buffers of pH values of 6.5, 7.0, 7.5, or 8.0, and the binding ratios were examined after 60 min. The results obtained at 32 and 0.4 mM iodoacetate are shown in Figure 3. The enzyme activity was fully maintained in all conditions. It will be seen that about two reactive groups were masked at pH values of 6.5 to 7.0 with the higher concentration of iodoacetate and that a third group reacted at pH 8.0. The binding ratio increases very sharply at higher pH values but remains fairly constant in the lower pH range. The same tendency was observed at the higher pH with the lower concentration of iodoacetate (0.4 mM). The higher pH may accelerate not only the reaction rate but also cause changes in the enzyme structure that lead to the exposure of the third reactive group.

TABLE I: Effect of Substrates on Iodoacetate Binding in the Presence of Glutamine.[a]

Further[b] Additions	Binding Ratio ([^{14}C]Iodoacetate: Enzyme) (mol/mol)	Rel Residual Act. (%)
None	2.2	108
FGAR	2.1	99
ATP	1.9	99
$MgCl_2$	1.9	101
FGAR, ATP	2.1	98
FGAR, $MgCl_2$	2.3	100
ATP, $MgCl_2$	2.2	99
FGAR, ATP, $MgCl_2$	1.9	98

[a] The complete substrate system contained in 0.1 mL: 0.43 nmol of FGAR amidotransferase, 100 mM glutamine, 0.43 mM FGAR, 5 mM ATP, 5 mM $MgCl_2$, 100 mM potassium phosphate (pH 7.2), and 32 mM [^{14}C]iodoacetate. The reaction mixture was incubated at 25 °C for 60 min, and then activities and binding ratios were measured. Activity is shown as percent of the activity measured at 0 time. [b] Additions to the basic system, which was comprised of enzyme, glutamine, and iodoacetate.

Effect of Substrates on Iodoacetate Binding. Most of the experiments mentioned above were carried out in a system containing only glutamine as substrate. To check the influence of all of the substrates, the enzyme was incubated with iodoacetate in potassium phosphate buffer (pH 7.2) containing various combinations of substrates at 25 °C for 60 min. The concentrations of the substrates were 0.43 mM FGAR, 5 mM ATP, and 5 mM $MgCl_2$. No significant differences were observed in any combination, the average binding ratio of iodoacetate to the nonessential groups in terms of mol/mol of enzyme being about 2 as shown in Table I. Taking account of all the results mentioned above, we recommend the following conditions as optimal for masking the two nonessential reactive groups without loss of enzyme activity: incubation for 60 min at 25 °C in a medium of pH 6.5–7.0 containing 24 mM iodoacetate and 100 mM glutamine.

Inactivation of FGAR Amidotransferase by Iodoacetate in the Absence of Glutamine

Effect of Substrates. Figure 4 confirms the previous observation (Schroeder et al., 1969; Mizobuchi et al., 1968) that addition of FGAR, ATP, and $MgCl_2$ in combination has a

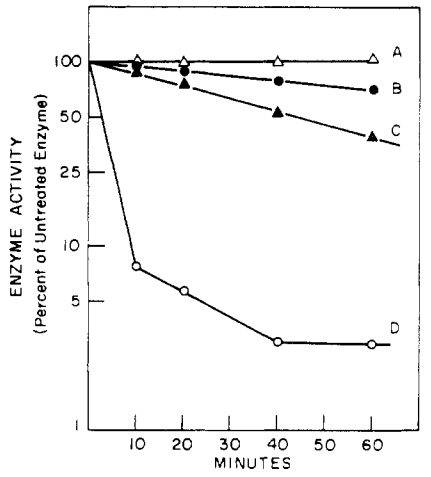

FIGURE 4: Effect of cosubstrates on inactivation of enzyme by iodoacetate in the absence of glutamine. FGAR amidotransferase (0.10 nmol) was incubated with potassium phosphate (50 mM) (pH 7.4), iodoacetate (2 mM), and various combinations of substrates (4.2 mM FGAR, 50 mM ATP, and 50 mM $MgCl_2$ in the complete system) in a total volume of 0.1 mL at 23 °C for 60 min. At specified times aliquots were removed for assay of enzyme activity. Conditions of incubation in the presence of iodoacetate: (A) no substrates and no iodoacetate as a control; (B) no substrates; (C) ATP and $MgCl_2$ present; (D) FGAR, ATP, and $MgCl_2$ present.

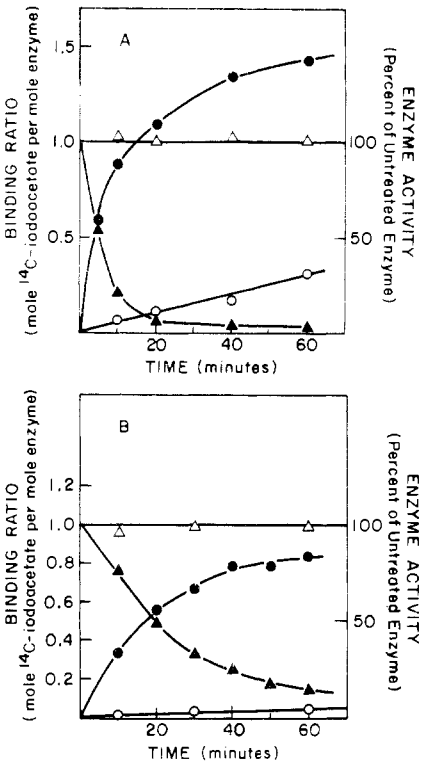

FIGURE 5: Inactivation and binding of masked enzyme by [^{14}C]iodoacetate during the specific labeling procedure: (A) (specific labeling at 25 °C) masked FGAR amidotransferase (160 nmol) was incubated with 80 mM potassium phosphate (pH 7.2), FGAR (0.1 mM), ATP (5 mM), $MgCl_2$ (5 mM), and [^{14}C]iodoacetate (0.5 mM) in a volume of 18 mL at 25 °C for 60 min (●, binding ratio: ▲, activity); as a control experiment, the masked enzyme (8.90 nmol) was incubated under the same conditions except with 12.5 mM glutamine present in a volume of 1 mL (○, binding ratio; △, activity). (B) (Specific labeling at 4 °C by improved method) masked FGAR amidotransferase (125 nmol), which had been previously treated after dialysis for the second time in a volume of 17.3 mL with unlabeled iodoacetate (0.2 mM) for 40 min at 25 °C in the absence of all substrates but with potassium phosphate buffer (pH 6.7) (100 mM), was then incubated with FGAR (0.43 mM), ATP (5 mM), $MgCl_2$ (5 mM), and with [^{14}C]iodoacetate to make a final concentration of 1.15 mM in a volume of 22.3 mL at 4 °C for 60 min (●, binding ratio; ▲, activity). As a control experiment the same sample (0.42 nmol) was incubated under the same conditions except with glutamine (92.5 mM) in a volume of 0.1 mL (○, binding ratio; △, activity).

significant influence on the rate of inactivation of the unprotected enzyme by iodoacetate. This rate of inactivation (curve D) is about 60-fold greater than that observed in the absence of cosubstrates when compared with the control system (Figure 4, curve B). The combination of ATP and Mg^{2+} without FGAR also enhances the rate threefold (curve C).

Effect of Temperature and Concentration of Iodoacetate. The rate of inactivation of the enzyme in the presence of FGAR, ATP, and $MgCl_2$ but in the absence of glutamine is a function of both temperature and concentration of iodoacetate. At a concentration of 0.2 mM iodoacetate the inactivation is very fast at 37 °C with a half-inhibition time of 3 min, moderate at room temperature with more than 90% inhibition after 60 min, and very slow at 0 °C with a half-time of 88 min. At a higher concentration of iodoacetate (1.15 mM) approximately 84% of the activity is lost by 60 min at 4 °C.

In the absence of all substrates there is insignificant inactivation of the enzyme at 24 °C, pH 7.2, and a concentration of iodoacetate of 0.2 mM during a 60-min incubation period. This differential in the activation rate observed in the presence and absence of FGAR, ATP, and $MgCl_2$ has been an essential factor in the labeling of the enzyme specifically at the glutamine site with radioactive iodoacetate.

Specific Labeling of Masked Enzyme

Specific Labeling at 25 °C. The masked enzyme was prepared by treating the native enzyme with 24 mM iodoacetate in the presence of 25 mM glutamine at 25 °C for 60 min. It was then dialyzed first against 0.1 M potassium phosphate buffer (pH 6.5) containing 10% glycerol and 25 mM glutamine for 5 h, and then against 0.1 M potassium buffer (pH 6.5) containing 10% glycerol at 4 °C for 30 h with several changes of buffer. This glutamine-free masked enzyme was next labeled with 0.5 mM radioactive iodoacetate in the presence of FGAR, ATP, and Mg^{2+} at 25 °C for 60 min.

Figure 5A shows an example of the inactivation and binding of radioactive iodoacetate to the masked enzyme at 25 °C. It was observed that some radioactive iodoacetate becomes bound to the enzyme in the control vessel in which reaction of the masked enzyme occurred in the presence of saturating glutamine. Since there was no loss of enzyme activity, no further binding of iodoacetate was expected in this control vessel. The amount bound in the presence of glutamine was variable depending on the preparation of the carboxymethylated enzyme, but usually it did not exceed 15–35% of the radioactive iodoacetate bound in the absence of glutamine. The binding ratio in the control experiment reached about 0.2 to 0.4 mol/mol of enzyme. The amount was sometimes too large to neglect, although occasionally it was very small. This phenomenon was observed whether the masked enzyme was treated with [^{14}C]iodoacetate in the presence of glutamine alone or with addition of ATP and Mg^{2+}. Therefore, this additional binding seems to result from the incidental exposure of new reactive groups during dialysis. Moreover, the binding ratio at the conclusion of the specific labeling step exceeded 1.0 at the time when the enzyme was completely inactivated. In a separate experiment (not shown) the binding ratio increased gradually to 2 when the incubation was continued longer.

Specific Labeling at 4 °C. To minimize nonspecific labeling, the method for specific labeling was modified as follows: the

masked enzyme was prepared and dialyzed as described above. It was then incubated again with 0.2 mM unlabeled iodoacetate at 25 °C for 40 min and the vessel was cooled in an ice bath for 30 min, conditions under which the activity was fully maintained even in the absence of glutamine. Then FGAR, ATP, $MgCl_2$, and [^{14}C]iodoacetate (to make a final concentration of 1.15 mM) were added to the vessel, and the incubation was continued at 4 °C for 60 min. A typical result is shown in Figure 5B. The binding of iodoacetate was 0.84 mol/mol of enzyme at 60 min and inactivation was 85% at this time. The binding in the control vessel containing glutamine was negligible. In a separate experiment in which the incubation was prolonged the binding ratio did not change for several hours after reaching a maximum. Therefore, we conclude that only the glutamine binding site was labeled by radioactive iodoacetate under these conditions.

Discussion

The experiments clarifying the conditions of binding of iodoacetate to FGAR amidotransferase have clearly shown that the glutamine-protected enzyme has several reactive groups that can be stepwise carboxymethylated but are not essential for activity. The binding was affected by the concentration of iodoacetate and by the temperature, pH, and time of incubation. As a rule the reaction proceeds in a more specific manner at a lower temperature (25 °C) and lower pH (6.5). We believe that the enzyme has two reactive but nonessential groups exposed on the surface of the protein and that others are buried in the tertiary structure. During incubation the structure gradually unfolds to expose new reactive groups, which will be subject to carboxymethylation. Possibly a conformational or denaturing change takes place not only under the conditions of incubation, such as elevated temperature and pH, but also by modification of enzyme with iodoacetate. Glutamine seems to protect the enzyme by forming a complex at the active site.

Upon removal of glutamine by dialysis at 4 °C, the masked enzyme may be reacted with [^{14}C]iodoacetate to label the residue at the active site. This reaction is greatly accelerated in the presence of FGAR, ATP, and $MgCl_2$ probably because the combination of these compounds leads to a change in the structure of the enzyme and the exposure of the reactive residue. This property of the enzyme has permitted the reaction of approximately 1 mol of [^{14}C]iodoacetate per mol of enzyme provided certain precautions are taken. During the relatively long dialysis period at 4 °C to remove glutamine from the masked enzyme, further nonessential groups become exposed. These groups must first be carboxymethylated with unlabeled iodoacetate before addition of the radioactive reagent together with FGAR, ATP, and $MgCl_2$.

All but one of the nine samples of enzyme labeled with [^{14}C]iodoacetate were labeled according to the procedure described in the section entitled Specific Labeling at 25 °C. However, we are fortunate that many of the samples showed little of the nonspecific incorporation of [^{14}C]iodoacetate. Furthermore, as pointed out in the accompanying paper (Ohnoki et al., 1977), we were able to identify the area of the glutamine active site labeled with iodoacetate by correspondence of amino acid sequences of azaserine-labeled peptides determined in previously reported experiments (Dawid et al., 1963; French et al., 1963).

As will be shown in the accompanying paper (Ohnoki et al., 1977) the tryptic peptides isolated from enzyme labeled with [^{14}C]iodoacetate contained only [^{14}C]carboxymethylcysteine. This observation serves to confirm our conviction that the equivalent of one sulfhydryl group is labeled at the glutamine active site. Keeping in mind that in the case of ribonuclease 1 equiv of histidine divided between two histidine residues may be carboxymethylated (Crestfield et al., 1963), we do not rule out the possibility that more than one sulfhydryl group may be present at the glutamine active site.

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