Functional arginyl residues as ATP binding sites of glutamine synthetase and carbamyl phosphate synthetase

(enzyme modification/phenylglyoxal/γ-glutamyl transfer/carbonyl phosphate/arginine)

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ABSTRACT The reaction of phenylglyoxal with two enzymes in which ATP plays a complex role has been studied. Both ovine brain glutamine synthetase and Escherichia coli carbamyl phosphate synthetase [carbamoyl-phosphate synthase (glutamine); ATP:carbamate phosphoryltransferase (dephosphorylating, amidotransferring); EC 2.7.2.9] were inactivated by phenylglyoxal. The specificity of this reagent for arginyl residues of the two proteins was confirmed by amino acid analysis. ATP, but not the other substrates, protected these enzymes against inactivation by phenylglyoxal. Carbamyl phosphate synthetase was also protected by IMP and ornithine, positive allosteric effectors that alter the enzymatic activity by increasing the affinity for ATP. UMP, a negative allosteric effector that decreases the affinity for ATP, did not protect against inactivation. Differential labeling experiments with [14C]phenylglyoxal showed that the number of arginyl residues protected by ATP corresponded quite well to the known number of ATP catalytic sites for each protein. These data indicate that arginyl residues at the active sites of glutamine synthetase and carbamyl phosphate synthetase are involved in the binding of ATP.

This phenylglyoxal inactivation study also provided information about the mechanistic role of ATP in the two synthetases. The data obtained on glutamine synthetase support the theory that ATP is attached to the enzyme as a portion of the catalytic site, and that its presence is essential for the binding of glutamate and glutamine. The data obtained on carbamyl phosphate synthetase are consistent with the previous proposal that carbonyl phosphate is an intermediate in the ATP-dependent activation of bicarbonate by this enzyme.

It is also of interest that, with both glutamine synthetase and carbamyl phosphate synthetase, only a small portion of the total arginyl population of these enzymes reacted with phenylglyoxal. A summary of previous studies on the modification of enzyme arginyl residues is presented.

Chemical modification of specific amino-acid residues within enzymes has provided much information on the role of individual residues in enzyme structure and function. Although the catalytic roles of many amino-acid residues have been elucidated by this approach, only recently has it been possible to selectively modify arginyl residues in such a manner as to allow exploration of their potential catalytic function. The procedure involves the reaction of an α-carbonyl compound with the arginyl guanidino group; the reagents that have been used include phenylglyoxal (1), 2,3-butanedione in borate buffer (2), and 1,2-cyclohexanedione in borate buffer (3). Thus, arginyl residues were identified as components of DPN binding sites in the alcohol dehydrogenases from human liver, horse liver, and yeast (4), and it was proposed that essential arginyl residues may be a general feature of the binding sites for DPN and for other anionic nucleotide coenzymes. Subsequently, essential arginyl residues have been identified at the DPN binding site of mitochondrial malate dehydrogenase (5) and at the ATP binding site of creatine kinase (6).

The present work is a study of the reaction of phenylglyoxal with two ATP-utilizing enzymes, ovine brain glutamine synthetase and Escherichia coli carbamyl phosphate synthetase [carbamoyl-phosphate synthase (glutamine); EC 2.7.2.9]. The role of ATP in these synthetases is more complex than in the direct phosphoryl transfer reaction catalyzed by creatine kinase (7). Thus, the identification of essential arginyl residues at the ATP binding sites of these enzymes would not only extend the generality of arginyl involvement in ATP binding but might permit the selective manipulation of essential arginyl residues in these enzymes, and thus constitute a new tool for studying mechanisms that involve substrate activation, phosphoryl transfer, and allosteric phenomena.

Glutamine synthetase catalyzes the following reaction:

\[
\text{i-glutamate} + \text{ATP} + \text{NH}_4^+ \xrightarrow{M^+} \text{i-glutamine} + \text{ADP} + \text{Pi} \quad [1]
\]

A number of data on the action of glutamine synthetase can be explained by a scheme (8) whose major features are the intermediacy of enzyme-bound γ-glutamyl phosphate and the attachment of ATP (or ADP) to the enzyme as part of the active site.

The overall reaction catalyzed by carbamyl phosphate synthetase is as follows:

\[
\text{i-glutamine} + 2 \text{ATP} + \text{HCO}_3^- \xrightarrow{K^+} \text{carbamyl phosphate} + 2 \text{ADP} + \text{Pi} + \text{i-glutamate} \quad [2]
\]

The enzyme can also catalyze two partial reactions which seem to reflect discrete steps in the synthesis reaction; these steps are thought to involve different ATP binding sites (9):

\[
\text{ATP} + \text{H}_2\text{O} \xrightarrow{\text{HCO}_3^-} \text{ADP} + \text{Pi} \quad [3]
\]

\[
\text{ADP} + \text{NH}_4\text{CO}_2\text{PO}_4^{2-} + \text{H}_2\text{O} \xrightarrow{\text{K}^+} \text{NH}_4^+ + \text{HCO}_3^- + \text{ATP} \quad [4]
\]

Presumably, reaction 3 corresponds to the activation of bi-

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carbonate by ATP, and reaction 4 reflects the reversal of the phosphorylation of enzyme-bound carbamate by ATP. The saturation curves for ATP in reaction 2 and ADP in reaction 4 are sigmoidal (9), and can be shifted to higher or lower nucleotide concentrations by allosteric effectors (10). Other features of the structure and function of carbamyl phosphate synthetase have been reviewed by Trotta et al. (11).

The present results strongly suggest that the ATP site of glutamine synthetase and both ATP sites of carbamyl phosphate synthetase contain functional arginy1 residues. In addition, the data provide information about the mechanisms of action of these synthetases.

EXPERIMENTAL

Materials. Ovine brain glutamine synthetase (12) and E. coli carbamyl phosphate synthetase (13) were isolated as previously described; the respective specific activities were 190 and 170 units/mg. Phenylglyoxal monohydrate was purchased from Aldrich Chemical Co. [7-14C]Phenylglyoxal (specific activity 1.6 X 10^6 cpm/mole) was prepared from [7-14C]acetophenone (ICN Corp.) by selenium oxidation (14). ADP, ATP, inosine monophosphate (IMP), uridine monophosphate (UMP), and lithium carbamyl phosphate were obtained from Sigma.

Methods. Glutamine synthetase activity was determined either by the γ-glutamyl hydroxamate synthesis assay (15) or by the γ-glutamyl transferase assay (16). The enzymatic activities of carbamyl phosphate synthetase were assayed as previously described (17); glutamine was used as the nitrogen donor in the assay for overall carbamyl phosphate synthetase activity. The decrease in enzymatic activity during reaction with phenylglyoxal was corrected for the decrease in activity of an unmodified control that was incubated under identical conditions. Thus, activity is expressed as the ratio of the activity of the modified enzyme, v, to that of the unmodified control enzyme, v0, multiplied by 100.

In studies on the binding of phenylglyoxal, the enzyme was incubated with [7-14C]phenylglyoxal and then assayed for enzymatic activity and for incorporation of radioactivity. The modified protein was separated from excess phenylglyoxal by gel filtration through a Sephadex G-25 column (1 X 10 cm) equilibrated with the buffer used for the incubation. Radioactivity was determined by liquid scintillation counting (18). Protein concentration was determined from absorbance at 280 nm, using molar absorptivities of 5.4 and 2.4 X 10^5 M^-1 cm^-1 for glutamine synthetase and carbamyl phosphate synthetase, respectively (12, 19). In the calculations, it was assumed that two phenylglyoxal molecules are bound per guanido group [see Takahashi (1)]. Amino-acid compositions were calculated from analyses performed in a Durrum 500 amino-acid analyzer; the samples were hydrolyzed in 6 N HCl in 60°C for 24 hr at 105°C.

RESULTS

Ovine brain glutamine synthetase

When glutamine synthetase was incubated with 4 mM phenylglyoxal, it was rapidly inactivated (Fig. 1; control). The presence of ATP plus MnCl2 protected the enzyme virtually completely. ATP plus MgCl2 afforded much less protection, as might be expected from the previous finding that the ATP-Mn++ complex is bound more tightly to the enzyme than the ATP-Mg++ complex (20). The presence of the metal ions alone also protected to some extent against inactivation; Mn++ was more effective than Mg++. Addition of ADP plus MnCl2 gave a pattern of protection that was almost identical to that found with ATP plus MnCl2. Glutamate (20 mM), however, neither prevented inactivation nor enhanced the protection afforded by the other substrates.

An enzyme binding study was performed with [7-14C]phenylglyoxal to determine the number of enzyme arginy1 residues that were modified in the presence and absence of ATP plus Mn++ (Table 1). Extrapolation of the results suggested that the enzyme contained 1.35 μM enzyme, 4 mM [7-14C]phenylglyoxal, 125 mM NaHCO3, and, as indicated, 20 mM MgCl2, 20 mM MnCl2, 20 mM MgCl2 plus 20 mM ATP, and 20 mM MnCl2 plus 20 mM ATP (final volume, 0.1 ml; final pH 7.9; incubated at 25°C). Samples (10 μl) were removed at the indicated time intervals and assayed for enzyme activity. Aliquots of control mixtures without phenylglyoxal were also assayed at time intervals, and these data were used to correct the phenylglyoxal inactivation values, as described in Methods.

Table 1. Differential labeling of glutamine synthetase and carbamyl phosphate synthetase with [14C]phenylglyoxal in the absence and presence of ATP

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity remaining (%)</th>
<th>Arginines modified (residues/subunit)</th>
<th>Arginines protected (residues/subunit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without ATP</td>
<td>With ATP</td>
<td>Without ATP</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>50</td>
<td>100</td>
<td>0.84</td>
</tr>
<tr>
<td>Carbamyl phosphate synthetase</td>
<td>50</td>
<td>95</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>90</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>80</td>
<td>12.8</td>
</tr>
</tbody>
</table>

The glutamine synthetase mixtures contained 1.35 μM enzyme, 4 mM [7-14C]phenylglyoxal, 125 mM NaHCO3, and, where indicated, 20 mM ATP plus 20 mM MnCl2 (final volume, 1.0 ml; final pH 7.9; incubated at 25°C). Samples were removed at 15 and 40 min, and assayed for activity (1 μl aliquot) and for phenylglyoxal binding (0.4 ml aliquot). The carbamyl phosphate synthetase mixtures contained 5.2 μM enzyme, 6 mM [7-14C]phenylglyoxal, 125 mM N,N-bis(2-hydroxyethyl) glycine, and, where indicated, 20 mM ATP plus 20 mM MgCl2 (final volume, 1.0 ml; final pH 7.9; incubated at 25°C). Samples were removed at 30 and 60 min, and assayed for overall carbamyl phosphate synthetase activity (2 μl aliquot) and for phenylglyoxal incorporation (0.4 ml aliquot).
numbers of arginyl residues modified at intermediate degrees of inactivation to 100% inactivation and to 100% protection showed that ATP plus Mn**+ protects 0.84–1.5 arginyl residues per subunit of glutamine synthetase.

Since only three of the enzyme arginyl residues out of the total of twenty-five (8) were modified in the completely inactivated protein, values for arginyl disappearance early in the time course could not be determined accurately. However, amino acid analysis of native glutamine synthetase and glutamine synthetase 95% inactivated by treatment with phenylglyoxal showed a 15% loss of arginyl residues. The analyses for all other amino-acid residues were identical for the native and inactivated enzymes, indicating that no amino acid other than arginine was modified.

**Carbamyl phosphate synthetase**

Incubation of carbamyl phosphate synthetase with 6 mM phenylglyoxal led to inactivation of the overall carbamyl phosphate synthetase activity and both partial activities, i.e., bicarbonate-dependent ATPase, and ATP formation from ADP and carbamyl phosphate (Exp. 1, Table 2). Under these conditions, the activities decreased at exponential rates for at least 30 min. The ATPase activity and the overall carbamyl phosphate synthetase activity were 50% inactivated in 8 min. The half-time for inactivation of the ATP formation activity was 15 min.

Addition of ATP plus MgCl₂ partially protected both the overall carbamyl phosphate synthesis and ATPase activities, while ADP plus MgCl₂ provided excellent protection of the ATP formation activity (Exps. 2 and 3). IMP and L-ornithine act as positive allosteric effectors of carbamyl phosphate synthetase by increasing the affinity of the enzyme for ATP (10, 21). These effectors partially protected all three activities against inactivation by phenylglyoxal, presumably by altering the conformation of the enzyme at the ATP sites (Exps. 4 and 5). It is notable that UMP, a negative effector that lowers the affinity for ATP (10), protected none of the activities (Exp. 6). Sodium bicarbonate at a high concentration increased the rate of inactivation of ATPase activity, and of overall carbamyl phosphate synthesis activity, while the ATP formation activity was unaffected (Exp. 7). A lower level of bicarbonate (Exp. 8) affected none of the activities. The two partial activities are further distinguished by the fact that carbamyl phosphate protected only the ATPase activity (Exp. 9). The glutamine site of the enzyme is apparently not directly involved in the inactivation by phenylglyoxal; thus, glutamine did not protect against phenylglyoxal (Exp. 10), and the rates of inactivation of the overall carbamyl phosphate synthesis reaction were identical with either glutamine or ammonia as the nitrogen donor.

To quantitate the arginine modification, carbamyl phosphate synthetase was incubated with [7-14C]phenylglyoxal in the presence and absence of ATP plus Mg**++. The differential labeling data are shown in Table 1. Extrapolation of these data leads to a calculated difference between totally inactivated and totally protected carbamyl phosphate synthetase of 3.0–3.6 modified residues per subunit. Comparison of amino-acid analyses of the native enzyme and of carbamyl phosphate synthetase inactivated to 25% of the original activity showed that only arginyl residues were lost upon modification. There was a 10% decrease in the amount of arginine, which corresponds well to the loss of 13 of the 90 arginyl residues (22).

**DISCUSSION**

From this study of phenylglyoxal inactivation, it may be concluded that arginyl residues are components of the ATP binding sites in both glutamine synthetase and carbamyl phosphate synthetase. Both enzymes were protected against phenylglyoxal inactivation by ATP, but not by other substrates. Carbamyl phosphate synthetase was also protected by effectors that are known to alter the affinity for ATP, presumably by changing the protein conformation at the ATP binding sites. In addition, differential labeling experiments showed that the number of arginyl residues protected by ATP corresponded closely with the number of ATP sites in each enzyme. The existence of one essential arginyl residue per subunit of glutamine synthetase is consistent with the previous demonstration of one tight-binding ATP site per subunit (23).
With carbamyl phosphate synthetase, it appears that phenylglyoxal modifies one essential arginyl residue at each of the two catalytic ATP sites, and that it also modifies a third arginyl residue that is protectable by ATP. The latter residue may be a component of the allosteric binding site for IMP. Thus, a positively charged arginyl residue would be complementary to the anionic ligand IMP, and ATP, acting as an IMP analogue, might therefore bind to this site. It is possible that the essential arginyl residues are located at sites on the proteins that are distinct from the ATP binding sites, and that in the presence of ATP they become inaccessible to phenylglyoxal because of conformational changes induced by the binding of ATP. However, the failure of other substrates to afford protection from inactivation and the numerical equivalence of arginyl residues protected by ATP and the number of ATP sites makes this interpretation less likely.

A mechanistic scheme has been proposed for glutamine synthetase that involves the formation of a γ-glutamyl phosphate intermediate and the attachment of ATP (or ADP) to the enzyme as a portion of the catalytic site required for the binding of glutamate and glutamine (8). This scheme is based partly on the observation that glutamine synthetase can catalyze transfer of the γ-glutamyl group from glutamine to hydroxylamine and that this transfer reaction requires catalytic amounts of ADP (or ATP). During phenylglyoxal inactivation of the enzyme, there is a parallel loss of both glutamine synthetase activity and γ-glutamyl transferase activity, and the two activities are protected equally well by ATP. Since the differential labeling data indicate that only one arginyl residue per subunit is required for activity and is protected by ATP, this arginyl residue must be a component of a single ATP binding site that is utilized during both the synthesis reaction and the transfer reaction. Thus, the inactivation data establish that the nucleotide required for the γ-glutamyl transferase reaction is bound at the catalytic ATP site, and thereby add support to the proposed mechanism of action.

It has been proposed that carbonyl phosphate (HOOC-PO$_3^{-2}$) is formed as an intermediate for the carbamyl phosphate synthetase reaction at the step of activation of bicarbonate by ATP (24). The fact that carbamyl phosphate, an analogue of carbonyl phosphate, partially protected only the ATP site involved in bicarbonate activation is consistent with the intermediate formation of carbonyl phosphate in this reaction. Carbonyl phosphate has also been proposed as an intermediate in the ATP-dependent carboxylation of biotin by bicarbonate (25), and it has been found (26) that biotin carboxylase can utilize carbamyl phosphate to carry out a reversal of the activation process; that is, the enzyme can catalyze the formation of ATP from ADP and carbamyl phosphate.

It is of interest that phenylglyoxal reacted with only a small number of the total arginyl residues, 3 out of 25 in glutamine synthetase, and 20 out of 90 in carbamyl phosphate synthetase. Presumably, most of the highly polar arginyl groups would be exposed on the surfaces of the two proteins. A few of these exposed arginyl residues may be involved in polar interactions with the other amino acid residues that preclude reaction with phenylglyoxal. Others will be solvated and their modification might be rather slow. But clearly some arginyl groups are in a chemical environment, perhaps hydrophilic, that enhances their reactivity toward phenylglyoxal. It is also possible that this environment contributes to the specificity of nucleotide binding. This appears to be a general phenomenon. Thus, in the previous studies on modification of enzyme arginyl residues (summarized in Table 3), it can be seen that in most instances the modification was quite selective.

It was previously noted that arginyl residues are important in the binding of DPN to dehydrogenases, and it was suggested that arginyl residues might serve the same function in enzymes that require other phosphate-containing coenzymes such as TPN, FAD, coenzyme A, UDPG, and ATP (4). The present work and recent investigations on creatine kinase (6) support this suggestion. Preliminary studies have shown that hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase are inactivated by α-dicarbonyl reagents 5, thus providing additional evidence for the proposed function of enzyme arginyl residues. Recent work on alkaline phosphatase (27), aldolase (28), and staphylococcal nuclease (29) indicates that arginyl residues function in the binding of phosphorylated substrates as well as of coenzymes. There is also evidence that arginyl residues may play a role in the binding of substrate carboxyl groups (2). It seems remarkable that arginine plays such a versatile role in the binding of the cofactors and substrates of a wide range of enzymes. The chemical phenomena involved in the function of enzyme arginyl groups probably include its strong positive charge, its ability to form multiple hydrogen bonds (30), and perhaps other types of interaction.

5 C. L. Borders, Jr. and J. F. Riordan, unpublished data.
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