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Chemical Modification and Ligand Binding Studies with *Escherichia coli* Glutamate Synthase†

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ABSTRACT: The structure and function of *Escherichia coli* glutamate synthase were studied by ligand binding and chemical modification experiments. Binding of $NADP^+$ was to a single dinucleotide site per $\alpha\beta$ protomer with a K_d of approximately 5 μM. Phenylglyoxal modified an essential arginyl residue required for binding of $NADP^+$. *E. coli* glutamate synthase thus employs a single dinucleotide binding site which functions in the NADPH to flavin electron transfer for glutamine-dependent glutamate synthase and for direct reduction of 2-iminoglutarate by NADPH in the NH_3-dependent reaction. Binding of 2-oxoglutarate was complex. "Half of the sites" binding of 2-oxoglutarate ($K_d < 0.25$ μM) was obtained in the absence of glutamine. Binding to half of the sites was pH independent. In the presence of glutamine, the 2-oxoglutarate binding ratio was approximately 1 equiv per protomer (K_d = 2–3 μM) at pH 7.5. This binding was pH dependent and varied between 0.43 equiv per protomer at pH 6.7 and 2.3 equiv per protomer at pH 9.0. Correlation of half of the sites binding with negative cooperativity for 2-oxoglutarate saturation indicates the utilization of low-K_d 2-oxoglutarate sites for NH_3-dependent glutamate synthase. Binding of glutamine promotes a conformational change that exposes additional 2-oxoglutarate sites having a K_d of 2–3 μM which are utilized in the glutamine-dependent reaction. Chemical modification with pyridoxal 5′-phosphate caused inactivation of glutamine-dependent but not NH_3-dependent glutamate synthase. Inactivation was ascribed to modification of one to two lysyl residues per protomer by Schiff base formation. The essential lysyl residue has a role in the binding of glutamine.

Escherichia coli glutamate synthase [L-glutamine:2-oxoglutarate aminotransferase (reduced $NADP^+$ oxidizing), EC 2.6.1.53] is a non-heme iron–sulfur flavoprotein (Miller & Stadtman, 1972). The enzyme has an essential role in ammonia assimilation, particularly under conditions of low ammonia concentration (Tempest & Meers, 1970). Homogeneous enzyme has been isolated from *Escherichia coli* (Miller & Stadtman, 1972; Mäntsälä & Zalkin, 1976a), *Klebsiella aerogenes* (Trotta et al., 1974), and *Bacillus megaterium* (Hemmilä & Mäntsälä, 1978a,b). These enzymes appear similar and exhibit an $\alpha_4\beta_4$ subunit structure.

Glutamate synthase catalyzes a glutamine- and NH_3-dependent synthesis of glutamate as shown by eq 1 and 2.

$$\text{glutamine} + \text{2-oxoglutarate} + \text{NADPH} + H^+ \rightarrow 2\ \text{glutamate} + NADP^+ \quad (1)$$

$$NH_3 + \text{2-oxoglutarate} + \text{NADPH} + H^+ \rightarrow \text{glutamate} + NADP^+ \quad (2)$$

For the *E. coli* enzyme, the rate of NH_3-dependent glutamate synthesis is 5–7% of that of the amidotransferase reaction (Mäntsälä & Zalkin, 1976a). Treatments which release non-heme iron, acid-labile sulfur, and flavin completely inactivate the amidotransferase activity and concomitantly stimulate the NH_3-dependent activity approximately 5-fold (Mäntsälä & Zalkin, 1976b; Hemmilä & Mäntsälä, 1978a,b). Evidence has accumulated that the mechansim of reductive amination is different for amidotransferase and NH_3-dependent reactions (Mäntsälä & Zalkin, 1976b; Geary & Meister, 1977). Although the NH_3-dependent reaction is formally similar to that of glutamate dehydrogenase, the two enzymes are immunochemically unrelated, and their catalytic parameters are distinct (Mäntsälä & Zalkin, 1976b).

The present experiments were conducted to (a) determine if glutamine- and NH_3-dependent activities of glutamate synthase utilize the same substrate binding and catalytic sites and (b) determine the roles of residues essential for catalysis.

Experimental Procedures

Materials. Homogeneous glutamate synthase was prepared from *E. coli* as previously described (Mäntsälä & Zalkin, 1976a). Freshly prepared enzyme had a specific activity of

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14–18 units/mg. 2-Oxo[U-^{14}C]glutarate, L-[U-^{14}C]glutamine, and NaB^3H_4 were from New England Nuclear. Phenyl[2-^{14}C]glyoxal was from Research Products International Corp. [4-^{3}H]$NADP^+$ was prepared as described (Dallocchio et al., 1976). ^{14}C-Labeled 2-amino-4-oxo-5-chloropentanoate (chloro ketone)[1] and unlabeled chloro ketone were prepared in this laboratory by Dr. W. Higgins according to the methods of Khedouri et al. (1966) and Pinkus & Meister (1972).

Enzyme Assay Methods. Glutamate synthase glutamine-dependent (Mäntsälä & Zalkin, 1976a) and NH_3-dependent (Tso et al., 1980) activities were assayed as described. Concentrations of glutamate synthase were determined from the absorbance at 278 nm; $E_{278}^{0.1\%} = 1.29$ (Miller & Stadtman, 1972).

Ligand Binding. Ligand binding experiments were performed by using the gel filtration method of Hummel & Dreyer (1962) on 0.8 × 35 cm columns of Sephadex G50F. For determination of the specific radioactivity, the concentration of 2-oxo[U-^{14}C]glutarate was determined by assay with *E. coli* glutamate synthase, and [^{3}H]$NADP^+$ was assayed with yeast glucose-6-phosphate dehydrogenase. The lowest ligand concentrations that were employed were dictated by the specific radioactivities that were available and by the amount of binding. Glutamate synthase was dialyzed against 50 mM K-Hepes, pH 7.5, containing 1 mM EDTA before use. A molecular weight of 188 000 for the $\alpha\beta$ glutamate synthase protomer was used for calculations.

For each column, 100 mL of buffer was prepared which contained 50 mM K-Hepes, pH 7.5, 1 mM EDTA, radioactive ligand, and additions as indicated. Columns were equilibrated with 40 mL of buffer, and then enzyme samples (3–5 nmol) in 0.1 mL were applied and eluted with another 40 mL of buffer. Fractions of 1.0 mL were collected. Free ligand was determined from the base-line level of radioactivity of the equilibrated column. Bound ligand was determined from the level of radioactivity above the base line in the fractions which contained protein. Protein was determined from the absorbance at 278 nm. A correction was made for the absorbance of $NADP^+$ at 278 nm. Clear separation of the protein peak from the ligand-depleted trough was achieved, assuring that equilibrium was attained. Close agreement was obtained between the areas of the peak and trough when the area of the trough was corrected for dilution due to the volume in which the enzyme was applied. The recovered enzyme retained 95–100% of its initial activity. Enzyme was aged by incubation at 4 °C in 50 mM K-Hepes, pH 7.5, containing 1 mM EDTA for a period of from 3 to 5 days. This treatment resulted in the loss of 60–85% of the glutamine-dependent glutamate synthase activity due to oxidation of the active-site cysteine.

Binding data were plotted according to Scatchard (1949):

$$\gamma_T/L_f = -K_d^{-1}\gamma_T + n/K_d \quad (3)$$

where γ_T is the ratio of ligand bound per enzyme protomer ($\alpha\beta$), L_f is the concentration of unbound ligand, K_d is the dissociation constant, and n is the number of ligand binding sites per enzyme protomer. Observed values were fitted to eq 3 by linear regression. pH titration data were analyzed with a nonlinear least-squares computer program (Markley, 1973).

Chemical Modifications of Glutamate Synthase. Phenylglyoxal modifications were performed in 50 mM $NaHCO_3$ at 22 °C. In some experiments, unreacted reagents were removed from enzyme samples by centrifugation–gel filtration (Penefsky, 1977), and enzyme specific activity was determined. In other experiments, aliquots were diluted into appropriate assay mixtures.

Reaction of glutamate synthase with pyridoxal-P was performed in 50 mM potassium phosphate, pH 7.5 at 22 °C. In experiments with $NaCNBH_3$ or NaCN to stabilize Schiff base adducts, reactions were initiated by addition of pyridoxal-P to the complete incubation mixture. Samples were removed and either diluted 50–100-fold into an assay mixture or treated by centrifugation–gel filtration. Incorporation of pyridoxal-P was determined by inorganic phosphate analysis for modifications utilizing $NaCNBH_3$ and by incorporation of radioactivity for those utilizing $Na^{14}CN$. When $NaBH_4$ was utilized, enzyme was incubated with pyridoxal-P for 20 min in a volume of 0.3 mL, and 0.30 mL of 0.1 M $NaBH_4$ was added. Excess reagents were removed by dialysis. Specific enzyme activity was determined after dialysis, and incorporation of pyridoxal-P residues was determined by the decrease of lysine by amino acid analyses.

Inactivation Kinetics. Inactivation data were initially plotted as the log of the percentage of the initial activity remaining vs. time. Second-order rate constants were determined as the slopes of linear plots of pseudo-first-order rate constants vs. the inactivating reagent concentration. Data were fitted by linear regression.

Anaerobic Reduction–Oxidation of Glutamate Synthase. Experiments were performed as previously reported (Mäntsälä & Zalkin, 1976b) with 2.4 μM native or chemically modified glutamate synthase in 1-mL anaerobic cuvettes. Incubation mixtures contained enzyme, 100 mM K-Hepes, pH 7.2, 2 mM 2-oxoglutarate, and 1 mM EDTA. Reduction was with either 2 μL of 0.1 M $Na_2S_2O_4$ or 5 μL of 0.02 M NADPH. Glutamine (2 mM) was added to initiate reoxidation.

Sucrose Density Gradient Sedimentation. Native or modified glutamate synthase (120 μg) and yeast alcohol dehydrogenase (15 μg) in 0.1-mL volume were layered onto 5-mL gradients of 5–20% sucrose (Martin & Ames, 1961) containing 50 mM buffer with 1 mM EDTA. The buffer was Tris-HCl with native enzyme and potassium phosphate with modified enzymes. Centrifugation in a Beckman SW 65 rotor at 26 000 rpm was for 16 h at 4 °C. Gradients were fractionated, and proteins were detected by the enzymatic activity or the absorbance at 278 nm. An M_r of 141 000 and an $s_{20,w}$ of 7.6 S were used for yeast alcohol dehydrogenase (Buhner & Sund, 1969).

Analyses. Amino acid analyses were performed with a Durrum D500 amino acid analyzer after 24-h hydrolysis in vacuo by azeotropic HCl. Ashings and inorganic phosphate analyses were performed as described by Ames & Dubin (1960).

Results

2-Oxoglutarate Saturation. Saturation of 2-oxoglutarate in the glutamine-dependent reaction exhibited Michaelis–Menten kinetics with an apparent K_m of 3 μM for 2-oxoglutarate, in agreement with previous work (Miller & Stadtman, 1972; Redina & Orme-Johnson, 1978). At pH 9.0, saturation of 2-oxoglutarate in the NH_3-dependent reaction exhibited negative cooperativity. Apparent K_m values of 2.7 and 360 μM were obtained from double-reciprocal plots at low (2.9–29 μM) and high (0.06–8.33 mM) 2-oxoglutarate concentrations, respectively.

2-Oxoglutarate Binding. In the absence of other ligands, with buffer near neutral pH, glutamate synthase bound approximately 0.5 equiv of 2-oxoglutarate per protomer (Figure

[1] Abbreviations: chloro ketone, 2-amino-4-oxo-5-chloropentanoate; pyridoxal-P, pyridoxal 5′-phosphate; Hepes, *N*-(2-hydroxethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

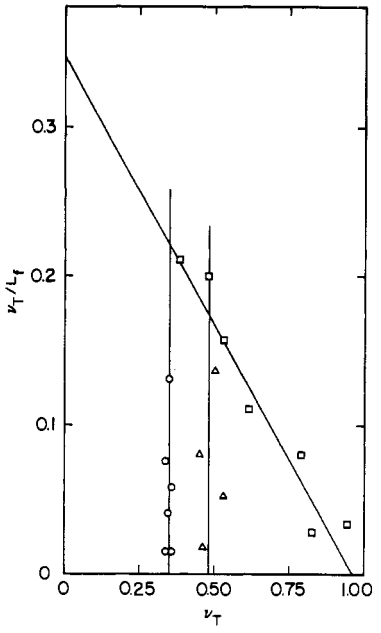

FIGURE 1: Scatchard plot for binding of 2-oxoglutarate to glutamate synthase. Elution buffers contained 2–20 μM 2-oxo[U-^{14}C]glutarate (○). To other elution buffers were added 0.2 mM NADP$^+$ (△) or 10 mM glutamine (□). γ_T is the ratio of ligand bound per $\alpha\beta$ enzyme protomer.

Table I: Binding of 2-Oxoglutarate to Fully Active or Aged Enzyme[a]

additions	sp act. (μmol min^{-1} mg^{-1})	n	K_d (μM)
none	4.3	0.49	<0.25
none	14.6	0.50	<0.25
10 mM glutamine	3.2	0.84	2.2
10 mM glutamine	15.3	0.96	2.7

[a] Binding of 2-oxo[U-^{14}C]glutarate was determined as described under Experimental Procedures. Additions to the elution buffer are noted.

1). A vertical line in the Scatchard plot suggests that the lowest concentration of ligand (2 μM) was saturating. The limit for K_d by graphical analysis is estimated at less than 0.25 μM. Addition of NADP$^+$ had no significant effect on 2-oxoglutarate binding. In the presence of 10 mM glutamine, the number of 2-oxoglutarate binding sites increased to 0.96 with a dissociation constant of 2.7 μM (Figure 1). NH_4^+ was without effect.

It was previously observed that glutamine-dependent glutamate synthase activity is labile due to facile oxidation of an active-site cysteinyl residue which is essential for formation of a glutaminyl–enzyme intermediate (Mäntsälä & Zalkin, 1976a). Aged enzyme forms a glutamine–enzyme complex but cannot form the acyl-enzyme covalent intermediate. By measuring the binding of 2-oxoglutarate to partially aged glutamate synthase, we were able to determine whether glutamine was needed to form an enzyme–substrate complex or the covalent enzyme intermediate. The data in Table I demonstrate that the 2-oxoglutarate binding ratio is dependent upon glutamine but is independent of amidotransferase specific activity. Thus, saturation of the 2-oxoglutarate sites requires binding of glutamine but not acyl-enzyme formation.

Glutamine- and NH_3-dependent glutamate synthase exhibits different pH optima (Tso et al., 1980). The effect of pH on 2-oxoglutarate binding was measured in order to determine whether full saturation of the 2-oxoglutarate site at pH 9.0 might replace the half of the sites binding obtained at pH 7.5.

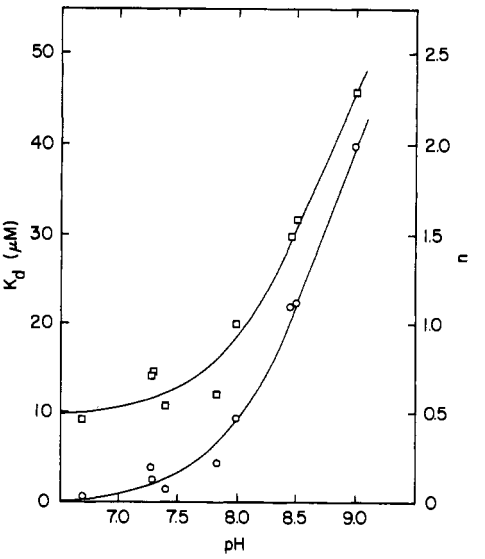

FIGURE 2: Glutamine-promoted pH-dependent 2-oxoglutarate binding. Binding was performed as described for Figure 1 except 50 mM Tris-HCl buffer of the indicated pH was substituted for K-Hepes. Values for K_d (○) and n (□) were obtained from Scatchard plots of binding data. The line is a computer fit to the binding data. The number of ligand binding sites, n, is expressed relative to the $\alpha\beta$ protomer.

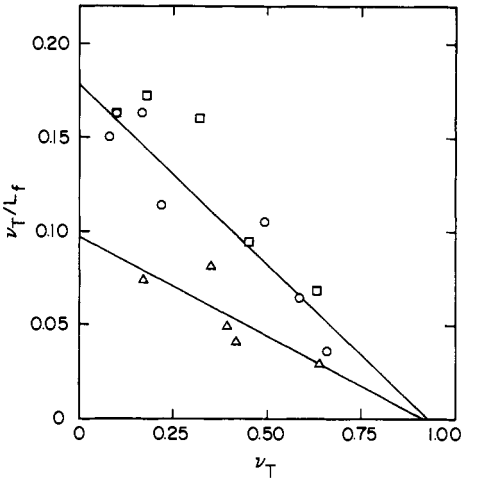

FIGURE 3: Binding of NADP$^+$ to glutamate synthase. Experiments were similar to those described in Figure 1 except that elution buffer contained 0.5–20 μM [4-^{3}H]NADP$^+$ instead of 2-oxo[U-^{14}C]glutarate. Scatchard plots show the binding of [4-^{3}H]NADP$^+$ in the absence of other ligands (○), [4-^{3}H]NADP$^+$ plus 2 mM 2-oxoglutarate (□), and [4-^{3}H]NADP$^+$ plus 10 mM glutamine (△). Lines were fit to the data for NADP$^+$ binding in the absence of other ligands and for binding with glutamine added.

Between pH values of 7.5 and 9.1, binding of 2-oxoglutarate in the absence of glutamine was similar to that shown in Figure 1. Thus, at pH 9.0, where NH_3-dependent activity is optimal, still only half of the sites binding was detected. The effect of pH on 2-oxoglutarate binding conducted in the presence of glutamine was very complex. As shown in Figure 2, both the extrapolated binding ratio and the K_d were pH dependent. The apparent binding ratio varied from 0.43 at pH 6.7 to 2.3 at pH 9.0. The K_d values varied from 0.5 μM at pH 6.7 to 39 μM at pH 9.0. A computer fit of the titration data yields pK_a values of 8.7 ± 0.2 and 8.8 ± 0.1 for the group(s) affecting n and K_d, respectively.

Sucrose gradient centrifugation at different pH values was used to determine if changes in the apparent binding constant reflect changes in the quaternary structure. A sedimentation coefficient of approximately 20 S, similar to that reported by

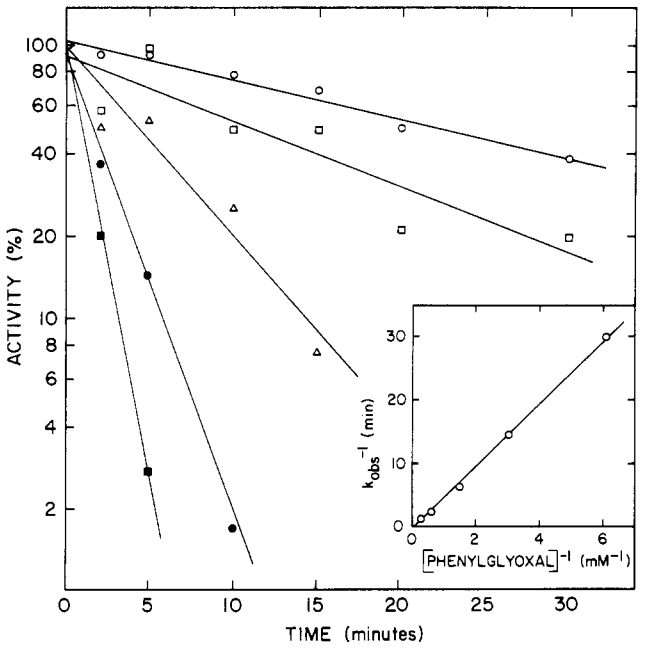

FIGURE 4: Inactivation of glutamine-dependent glutamate synthase by phenylglyoxal. Inactivations were performed as described under Experimental Procedures with 2.4 μM glutamate synthase and the indicated concentrations of phenylglyoxal. The reciprocals of pseudo-first-order rate constants (k_{obsd}) were plotted against reciprocal phenylglyoxal concentrations. Enzyme was inactivated with 0.17 (○), 0.33 (□), 0.67 (△), 1.67 (●), or 3.33 mM (■) phenylglyoxal.

Miller & Stadtman (1972), was obtained and was found to be independent of pH from pH 7.5 to 8.9.

NADP$^+$ Binding. The data in Figure 3 show that glutamate synthase bound NADP$^+$ with a stoichiometry of 0.9–1 equiv per protomer. The K_d calculated from Figure 3 is 5.2 μM. The addition of either 2 mM 2-oxoglutarate or 10 mM glutamine, or both (not shown), had no significant effect on NADP$^+$ binding. There was no apparent relationship between the loss of amidotransferase activity due to aging and the stoichiometry for NADP$^+$ binding. The binding stoichiometry was essentially the same at pH 7.5 and pH 9.0.

Arginyl Modification. Arginyl modification was investigated in view of its implication in binding anionic substrates (Kantrowtiz & Lipscomb, 1976; Takahashi, 1968) and coenzymes (Lange et al., 1974; Blumental & Smith, 1975). Preliminary experiments established that phenylglyoxal and butanedione inactivated glutamate synthase more effectively than cyclohexanedione. Phenylglyoxal was examined in more detail.

As shown in Figure 4, phenylglyoxal inactivated glutamate synthase glutamine-dependent activity with pseudo-first-order kinetics. Virtually identical inactivation was obtained for the NH_3-dependent activity. The initial rates of inactivation were proportional to the phenylglyoxal concentration over the range of 0.17–5 mM for each activity. Plots of pseudo-first-order rate constants vs. phenylglyoxal concentrations were linear as shown in the inset to Figure 4. Second-order rate constants of 217 and 162 M^{-1} min^{-1} were calculated for the inactivation of glutamine- and NH_3-dependent activities, respectively. The similarity in rate constants suggests that modification of the same residue is a major factor for inactivation of both reactions.

To localize the site of the essential arginyl modification, we examined the effect of substrates on modification by phenylglyoxal (Table II). NADP$^+$ protected against inactivation of glutamine-dependent glutamate synthase by phenylglyoxal, suggesting that an arginyl residue is required for dinucleotide

Table II: Protection against Phenylglyoxal Inactivation of Glutamate Synthase by NADP$^+$ and Its Analogues[a]

protecting reagent	inactivation half-time (min)
none	11
0.1 mM NADP$^+$	28
1.0 mM NADP$^+$	100
0.1 mM NAD$^+$	11
1.0 mM NAD$^+$	9
0.1 mM 2′-P-5′-ADP-Rib[b]	77
1.0 mM 2′-P-5′-ADP-Rib	86
0.1 mM 5′-ADP-Rib[c]	11
1.0 mM 5′-ADP-Rib	12
0.1 mM 2′,5′-ADP	18
1.0 mM 2′,5′-ADP	57
0.1 mM 2′-AMP	11
1.0 mM 2′-AMP	25
0.1 mM 5′-AMP	10
1.0 mM 5′-AMP	10

[a] Inactivation of glutamate synthase amidotransferase activity was with 0.5 mM phenylglyoxal. Half-times of inactivation were determined from plots of log percent activity vs. time. [b] 2′-Monophosphoadenosine 5′-diphosphoribose. [c] Adenosine 5′-diphosphoribose.

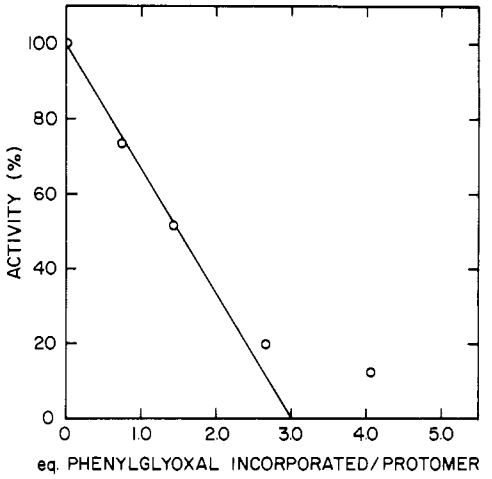

FIGURE 5: Relationship between glutamate synthase inactivation and phenylglyoxal incorporation. Phenyl[2-^{14}C]glyoxal was used to assay incorporation.

binding. Glutamine and 2-oxoglutarate were without effect on phenylglyoxal inactivation (data not shown).

To further define the role of the essential arginyl residue, we tested compounds containing portions of the NADP$^+$ molecule for the capacity to protect glutamate synthase glutamine-dependent activity from inactivation by phenylglyoxal (Table II). Significant protection was provided by 2′-AMP, 2′,5′-ADP, and 2′-P-ADP-Rib. P_i and a number of other nucleotides were without effect. These results suggest that the essential arginyl residue interacts with the 2′-phosphate of NADP$^+$.

Arginyl modification was quantitated in glutamate synthase that had been inactivated by phenyl[^{14}C]glyoxal. The data in Figure 5 show incorporation of phenyl[^{14}C]glyoxal into glutamate synthase plotted as a function of inactivation. Samples from an incubation mixture containing 2.6 μM glutamate synthase and 0.5 mM phenyl[^{14}C]glyoxal were alternately diluted 100-fold into the glutamine-dependent assay mixture to monitor the loss of activity and were treated by centrifugation–gel filtration to remove excess reagents so as to allow determination of phenylglyoxal incorporation. Extrapolation from 50% inactivation yields a stoichiometry of 3.0 equiv of phenylglyoxal incorporated per protomer for

Table III: Binding of $NADP^+$ to Native and Chemically Modified Glutamate Synthase[a]

enzyme	activity (%)	K_d (mM)	n
native	100	5.2	0.92
phenylglyoxal modified	10	2.4	0.09
pyridoxal-P–NaCN modified	<5	3.5	0.94

[a] Ligand binding and chemical modifications are described under Experimental Procedures. The concentration of [4-^{3}H]$NADP^+$ was 0.5–20 μM.

Table IV: Effect of Chemical Modifications on Activity and on 2-Oxoglutarate Binding[a]

enzyme	glutamine	activity (%)	K_d (μM)	n
native	–	100	<0.25	0.47
	+	100	1.4	0.86
phenylglyoxal modified	–	10	<0.25	0.44
	+	10	2.1	0.91
pyridoxal-P–NaCN modified	–	<5	<0.25	0.54
	+	<5	<0.25	0.44

[a] Ligand binding and chemical modifications are described under Experimental Procedures. The concentration of [4-^{3}H]$NADP^+$ was 0.5–20 μM.

complete inactivation. In a second experiment, protection by $NADP^+$ was used to determine the number of essential arginyl residues modified by phenyl[^{14}C]glyoxal. Phenylglyoxal incorporation was reduced approximately 2 equiv per protomer in glutamate synthase that had been protected by $NADP^+$.

It has been reported that phenylglyoxal inactivation of *B. megaterium* glutamate synthase was prevented by protection of the active-site cysteinyl residue with *p*-mercuribenzoate (Hemmilä & Mäntsälä, 1978b). Protection of the active-site cysteinyl residue in *E. coli* glutamate synthase with *p*-mercuribenzoate had no effect on the kinetics of inactivation of amidotransferase activity by phenylglyoxal (not shown).

$NADP^+$ and 2-Oxoglutarate Binding by Phenylglyoxal-Modified Enzyme. Direct evidence confirming that phenylglyoxal modified an essential arginyl residue at the $NADP^+$ site was obtained from binding experiments. Glutamate synthase was 90% inactivated by treatment with phenylglyoxal. The stoichiometry of $NADP^+$ binding to the inactivated enzyme was 0.09 equiv per protomer (Table III). The 90% reduction in binding capacity from 0.92 equiv per protomer for the native enzyme correlates with the extent of inactivation and is consistent with the conclusion that inactivation results from defective $NADP^+$ binding. The 10% residual $NADP^+$ binding exhibited a K_d similar to that obtained with native enzyme.

Direct binding measurements established that 2-oxoglutarate interacted normally with its site in phenylglyoxal-modified enzyme. The data in Table IV show that the 2-oxoglutarate binding stoichiometry and K_d for inactivated enzyme are equivalent to those of native glutamate synthase.

Anaerobic Reduction and Reoxidation of Flavin in Phenylglyoxal-Modified Enzyme. Although evidence has been presented that phenylglyoxal modified an arginyl residue required for binding the 2′-phosphate of $NADP^+$, it was important to determine whether this modification alters other glutamine amidotransferase functions. Previous experiments have established that glutamate synthase flavin can be reduced anaerobically by dithionite (Miller & Stadtman, 1972; Mäntsälä & Zalkin, 1976b; Geary & Meister, 1977). Furthermore, glutamine and 2-oxoglutarate can reoxidize the chemically reduced enzymes with the concomitant synthesis of glutamate. The data in Figure 6 show that phenylglyoxal-modified glutamate synthase was reduced normally with dithionite and was reoxidized normally by glutamine and 2-oxoglutarate. However, as shown in Figure 6, anaerobic reduction of flavin by NADPH was defective in the phenylglyoxal-modified enzyme. Flavin reduction was approximately 25% of that obtained with the native enzyme. This residual reduction is due to incomplete inactivation by phenylglyoxal. The secondary addition of dithionite caused complete reduction of the flavin in the modified enzyme. These experiments indicate that NADPH-mediated reduction of flavin is defective in the phenylglyoxal-modified enzyme, but subsequent steps in the amidotransferase mechanism leading to synthesis of glutamate occur normally.

Table V: Inactivation of Glutamate Synthase by Pyridoxal-P[a]

reagent to stabilize Schiff base	[pyridoxal-P] (mM)	amidotransferase activity (%)	lysine modified per protomer (equiv)
50 mM $NaBH_4$	0	100	0
	10	40	8.3
	80	3.5	19
10 mM $NaCNBH_3$	5	3	10
10 mM NaCN	5	6	1.6

[a] Glutamate synthase was inactivated by treatment with the indicated concentration of pyridoxal-P. Reactions with $NaCNBH_3$ were terminated by dialysis after 30 min, and those with NaCN were terminated by centrifugation–gel filtration after 20 min. Reaction conditions and methods for determination of pyridoxal-P incorporation are described under Experimental Procedures.

Lysyl Modification. A number of pyridine nucleotide dependent dehydrogenases are known to have a lysyl residue essential for activity, and a role in substrate or coenzyme binding has been implicated (Piszkiewicz & Smith, 1971; Chen & Engel, 1975; Bleile et al., 1976). Pyridoxal-P is a specific reagent for modification of the ϵ-amino groups of lysine residues by Schiff base formation (Piszkiewicz & Smith, 1971; Chen & Engel, 1975). Initial experiments demonstrated a time-dependent inactivation of glutamate synthase by pyridoxal-P. Rapid reversibility suggested that the Schiff base was unstable and required reduction by $NaBH_4$ or $NaCNBH_3$. Lability of the Schiff base precludes secondary X–azolidine formation (Wimmer et al., 1975; Bleile et al., 1976) with a nucleophile such as a cysteinyl residue. Various treatments were used to stabilize the initial Schiff base adduct, and representative data are shown in Table V. Nearly complete inactivation of glutamine–dependent glutamate synthase was obtained by reduction of the azomethine adducts by $NaBH_4$ or $NaCNBH_3$ or by addition of HCN to the carbon–nitrogen double bond. With $NaCNBH_3$ or NaCN to stabilize the Schiff base, 16-fold lower pyridoxal-P concentrations were capable of inactivation, comparable to that obtained by using $NaBH_4$. The advantages of reduction by $NaCNBH_3$ have been discussed (Jentoft & Dearborn, 1979). The selectivity of lysyl modification was improved by using the lower concentration of pyridoxal-P and $NaCNBH_3$ reduction. The greatest selectivity of lysyl modification was obtained with NaCN to stabilize the Schiff base; approximately 1–2 equiv of lysyl residues per protomer was modified. Enzyme inactivated by pyridoxal-P and any of the secondary treatments was not reactivated by dialysis against buffer solution containing mercaptoethanol.

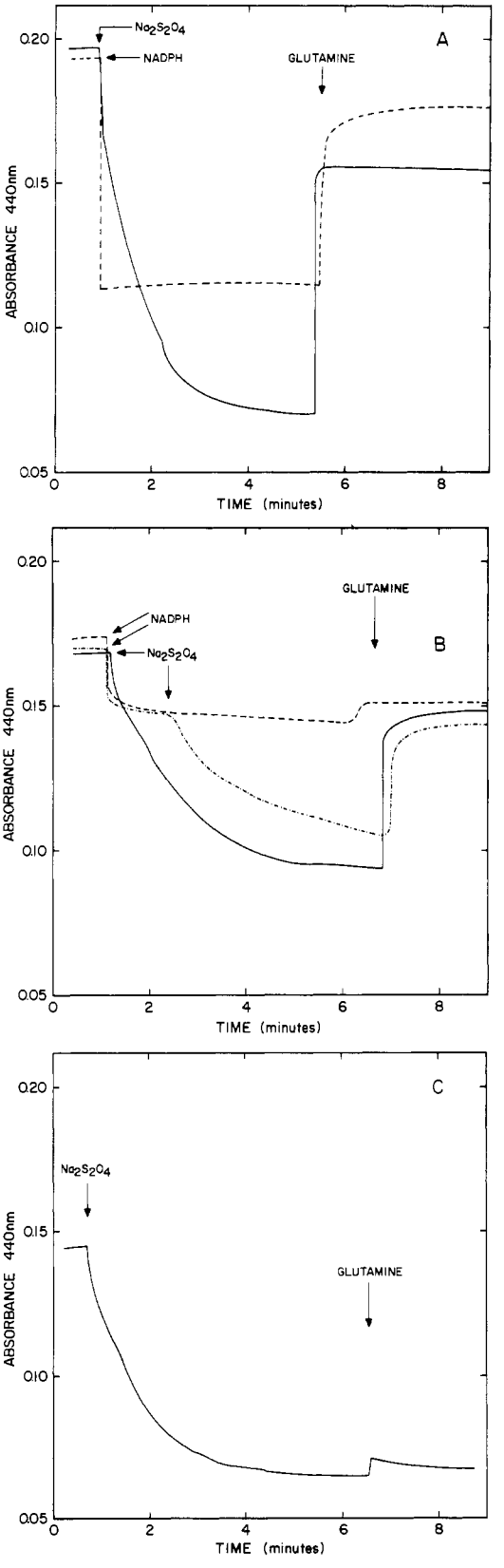

FIGURE 6: Anaerobic reduction–oxidation of native and chemically modified glutamate synthase. Details are given under Experimental Procedures. (A) Native glutamate synthase; (B) phenylglyoxal-modified glutamate synthase with 15% residual glutamine-dependent activity. In one experiment, enzyme (—) was initially reduced with $Na_2S_2O_4$. Two other samples of enzyme (- - - and –·–) were reduced with NADPH. At the indicated time, $Na_2S_2O_4$ was added to one of the NADPH-reduced enzymes (–·–). (C) Pyridoxal-P–$NaCNBH_3$-modified glutamate synthase with <5% residual glutamine-dependent activity.

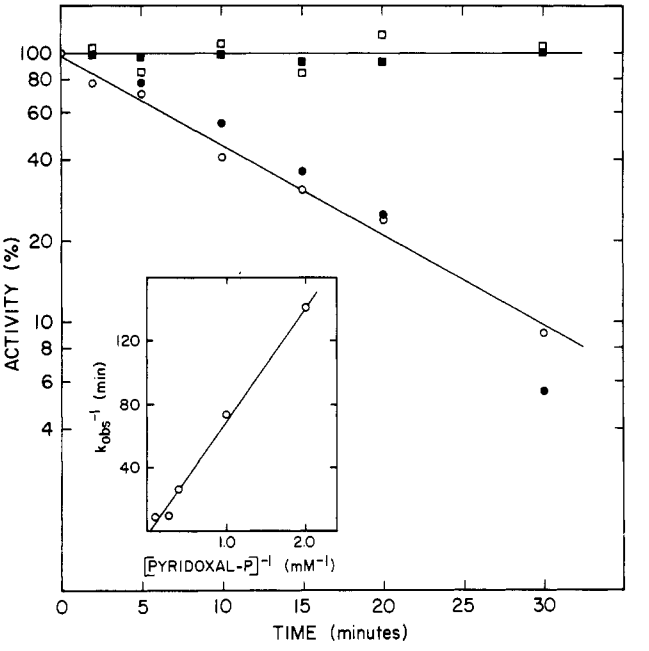

FIGURE 7: Inactivation of glutamate synthase by pyridoxal-P. Glutamate synthase (2.5 μM) was modified with 5 mM pyridoxal-P and 10 mM $NaCNBH_3$ or 10 mM NaCN. Glutamine-dependent activity was monitored for modification by using $NaCNBH_3$ (○) or NaCN (●) to stabilize the Schiff base. NH_3-dependent activity was monitored when modification was with $NaCNBH_3$ (□) or NaCN (■) to stabilize the Schiff base. Inset: Double-reciprocal plot of pseudo-first-order rate constants (k_{obsd}) vs. pyridoxal-P concentration for glutamine-dependent activity inactivation with $NaCNBH_3$.

Absorption Spectrum of Modified Enzyme. Glutamate synthase inactivated with pyridoxal-P and $NaCNBH_3$ or NaCN exhibited an absorption spectrum with a maximum at 325 nm (not shown). This spectrum was typical for that of a secondary amine resulting from reduction of the pyridoxal-P–lysyl Schiff base or aminonitrile adduct of the imine (Anker & Boehne, 1952). Taken together, these results support the view that pyridoxal-P inactivates glutamate synthase by Schiff base formation with essential lysyl residues.

Role of the Essential Lysyl Residue. Further experiments were conducted to determine the role of the essential lysyl residue(s). Modification using $NaCNBH_3$ or NaCN to stabilize the pyridoxal-P Schiff base adduct inactivated glutamine-dependent but not NH_3-dependent glutamate synthase (Figure 7). The inactivation exhibited pseudo-first-order kinetics. The inactivation was dependent upon the pyridoxal-P concentration (inset to Figure 7) but not upon the concentration of $NaCNBH_3$ or NaCN. The rate-determining step for inactivation therefore involves only enzyme and pyridoxal-P and is thus likely to be azomethine formation. The second-order rate constant for inactivation was 21 M^{-1} min^{-1}. Inactivation was dependent on the presence of pyridoxal-P; $NaCNBH_3$ and NaCN alone were not inhibitory.

Modification of the lysyl residue implicated in the glutamine amide transfer function was partially prevented by *O*-carbamylserine, a competitive inhibitor (K_i = 1.1 mM) with respect to glutamine. The rate of inactivation was reduced 5-fold by 2.5 mM *O*-carbamylserine. Serine was without effect. The effect of glutamine could not be accurately evaluated because of the enzyme's glutaminase activity. $NADP^+$ and 2-oxoglutarate at concentrations greater than 200-fold higher than their K_i and K_m values, respectively, did not influence inactivation by pyridoxal-P. Taken together, these results are consistent with modification of a lysyl residue specifically required for glutamine-dependent activity.

Further experiments were conducted to evaluate the role of the essential lysyl residue in glutamine-dependent glutamate synthase. We sought to distinguish between a role in (a) binding of glutamine, (b) activation of glutamine to form the covalent enzyme intermediate, or (c) amide transfer and hydrolysis of the covalent enzyme–substrate intermediate. Affinity labeling of the active-site cysteinyl residue with chloro ketone requires steps a and b (Mäntsälä & Zalkin, 1976a). Native glutamate synthase was affinity labeled with 25 μM ^{14}C-labeled chloro ketone for 30 min at 22 °C with incorporation of 0.74 equiv per protomer. Under identical conditions, glutamate synthase that had been modified with pyridoxal-P and $NaCNBH_3$ and dialyzed to remove excess reagents (95% inactivation) was affinity labeled by ^{14}C-labeled chloro ketone to the extent of 0.12 equiv per protomer. This result confirms that when the essential lysyl residue is modified either glutamine binding or formation of the covalent enzyme–substrate intermediate is blocked. Data summarized in Table IV evaluate glutamine binding by the pyridoxal-P-treated enzyme. The key result is that glutamine-independent binding of 2-oxoglutarate was normal in pyridoxal-P-modified glutamate synthase, but there was no glutamine-dependent stimulation of 2-oxoglutarate binding. Thus, the modified enzyme cannot bind glutamine. Further support for this conclusion was obtained from the anaerobic reduction and reoxidation of enzyme-bound flavin. The flavin in pyridoxal-P-modified glutamate synthase was normally reduced by dithionite (Figure 6C) or NADPH (not shown), but the normal reoxidation of reduced flavin by glutamine plus 2-oxoglutarate did not take place. Lysyl modification had no effect on $NADP^+$ binding (Table III) nor was the quaternary structure of the enzyme altered.

Discussion

E. coli glutamate synthase is a large and complex non-heme iron- and sulfur-containing flavoprotein. The purified enzyme is predominantly in the form of an $\alpha_4\beta_4$ oligomer of M_r approximately 800 000 (Miller & Stadtman, 1972). The $\alpha\beta$ protomer has an M_r of approximately 188 000. Due to its large size and functional complexity, there have been few detailed studies on the relationship of structure to function. We have utilized techniques of ligand binding and chemical modification to examine the structure and function.

Our studies have provided strong evidence for a single dinucleotide binding site per protomer that is utilized by NADPH for the reduction of flavin in the amidotransferase reaction and for the direct reduction of 2-iminoglutarate in the NH_3-dependent reaction. $NADP^+$ exhibits a K_d of approximately 5 μM which is in close agreement with the K_i for $NADP^+$ of 3.7 μM (Redina & Orme-Johnson, 1978) and with K_m values for NADPH of 7.7 and 14 μM for the glutamine- and NH_3-dependent reactions, respectively (Miller & Stadtman, 1972; Mäntsälä & Zalkin, 1976b).

Chemical modification of glutamate synthase with phenylglyoxal provided evidence for an essential arginyl residue. When phenyl[^{14}C]glyoxal was used, 2–3 equiv per protomer was required for total inactivation (Figure 5). Assuming reaction of 2 equiv of phenylglyoxal per arginyl residue (Takahashi, 1968), we estimate that modification of approximately 1–1.5 arginyl residues out of approximately 98 per protomer was required for complete inactivation. It appears likely that one arginyl residue per protomer is a component of the $NADP^+$ site. This residue interacts with the 2′-phosphate of the AMP moiety of the dinucleotide (Table II). Charge interaction of the 2′-phosphate of NADPH with an arginyl residue in dihydrofolate reductase has been determined by X-ray diffraction analyses (Matthews et al., 1979).

Modification of the essential arginine specifically reduced binding of $NADP^+$ (Table III) but not 2-oxoglutarate or glutamine (Table IV). Glutamine interaction was evaluated indirectly by its stimulation of 2-oxoglutarate binding. The specificity of the arginyl modification at the $NADP^+$ site is emphasized by experiments which show that other enzymatic functions were intact following modification. Anaerobic flavin reduction–oxidation experiments demonstrated that reduction of flavin by dithionite and reoxidation of reduced flavin by 2-oxoglutarate and glutamine were unaffected by arginyl modification (Figure 6). Reduction of flavin by NADPH was specifically blocked by arginyl modification.

Previous studies (Mäntsälä & Zalkin, 1976b; Geary & Meister, 1977) have established that glutamine- and NH_3-dependent glutamate synthase employ different mechanisms for reductive amination of 2-oxoglutarate. The intermediate formed from 2-oxoglutarate and glutamine is reduced by NADPH via flavin whereas the iminoglutarate formed from 2-oxoglutarate and NH_3 is directly reduced by NADPH. Our data demonstrate that NADPH bound to a single site can transfer electrons either to flavin or to α-iminoglutarate directly. In both reactions, the hydrogen atom at the B side of C_4 of the nicotinamide ring of NADPH is used (Geary & Meister, 1977).

2-Oxoglutarate exhibited complex binding. In the absence of glutamine, the 2-oxoglutarate binding stoichiometry was approximately 0.5 equiv per protomer. The K_d for this "half-site" binding was less than 0.25 μM. Saturation of NH_3-dependent glutamate synthase with 2-oxoglutarate at pH 9.0 exhibited negative cooperativity. Apparent K_m values of 360 and 2.7 μM were obtained at high and low levels of 2-oxoglutarate, respectively. Possibly, binding of 2-oxoglutarate to the low-K_m site corresponds to the half-sites detected by direct binding measurements. Half-site binding was independent of pH. Additional binding corresponding to the high-K_m saturation would not be detected if K_d and K_m values correspond.

In the presence of glutamine, a binding stoichiometry of approximately 1 equiv of 2-oxoglutarate per protomer was obtained at pH 7.5. On the basis of the similarity of the K_d (2.7 μM) with the K_m for 2-oxoglutarate, this site appears to function in the glutamine-dependent synthesis of glutamate. It is unclear whether the effect of glutamine is to relieve the half of the sites binding for 2-oxoglutarate and allow saturation of the two remaining sites in the $\alpha_4\beta_4$ oligomer or whether glutamine binding promotes a conformational change which masks half-site interaction and exposes a distinct 2-oxoglutarate site in each $\alpha\beta$ protomer. Either model predicts that 2-oxoglutarate binds to half of the sites for the NH_3-dependent synthesis of glutamate. The important distinction is whether identical or different 2-oxoglutarate sites are used for NH_3- and glutamine-dependent synthesis of glutamate. The effect of pH on glutamine-stimulated 2-oxoglutarate binding is pertinent to this question. The data summarized in Figure 2 show that glutamine-stimulated 2-oxoglutarate binding to the $\alpha\beta$ protomer varied from 0.43 equiv at pH 6.7 to 2.3 equiv at pH 9.0. It is thus apparent that half of the sites 2-oxoglutarate binding was detected even in the presence of glutamine, and at high pH, each protomer has the capacity to bind at least 2 equiv of 2-oxoglutarate. Although there are alternative possibilities, these data suggest that NH_3-dependent glutamate synthase utilizes 2-oxoglutarate sites that exhibit half of the sites binding and that glutamine exposes additional 2-oxoglutarate sites. We propose that the 2-oxoglutarate sites

used for the NH_3-dependent reaction can be fully saturated at pH 9.0 with high levels of substrate (K_m = 360 μM), but the high K_m precludes direct measurement by conventional binding experiments. These sites are saturated by lower 2-oxoglutarate concentrations (K_d of approximately 39 μM) in the presence of glutamine.

Inactivation of glutamate synthase with pyridoxal-P has provided evidence for an essential lysyl residue at the binding site of glutamine. Under the most favorable conditions, modification of approximately 2 lysyl residues per protomer out of a total of 69 lysyl residues resulted in greater than 90% inactivation. The most selective modification was obtained by using HCN to add to the ϵ-NH_2 pyridoxal-P azomethine for stabilization of the adduct. Of the enzymatic functions tested, only binding of glutamine was strongly inhibited by lysyl modification; $NADP^+$ binding, glutamine-independent binding of 2-oxoglutarate, reduction of flavin by NADPH and dithionite, and NH_3-dependent glutamate synthase were unaffected. All glutamine amidotransferases thus far examined utilize an active-site cysteinyl residue to activate glutamine. A lysyl residue essential for glutamine-dependent anthranilate synthase has recently been detected (Bower & Zalkin, 1982) and may represent a second residue required for glutamine amidotransferase function.

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Registry No. NADP, 53-59-8; NAD, 53-84-9; 2′-P-5′-ADP-Rib, 53595-18-9; 5′-ADP-Rib, 20762-30-5; 2′,5′-ADP, 3805-37-6; 2′-AMP, 130-49-4; 5′-AMP, 61-19-8; glutamine, 56-85-9; 2-oxoglutaric acid, 328-50-7; arginine, 74-79-3; lysine, 56-87-1; phenylglyoxal, 1074-12-0; pyridoxal-P, 54-47-7; glutamate synthase, 37213-53-9.

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