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L-Serine Binds to Arginine-148 of the β_2 Subunit of *Escherichia coli* Tryptophan Synthase†

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ABSTRACT: Inactivation of the β_2 subunit and of the $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli* by the arginine-specific dicarbonyl reagent phenylglyoxal results from modification of one arginyl residue per β monomer. The substrate L-serine protects the holo β_2 subunit and the holo $\alpha_2\beta_2$ complex from both inactivation and arginine modification but has no effect on the inactivation or modification of the apo forms of the enzyme. This result and the finding that phenylglyoxal competes with L-serine in reactions catalyzed by both the holo β_2 subunit and the holo $\alpha_2\beta_2$ complex indicate that L-serine and phenylglyoxal both bind to the same essential arginyl residue in the holo β_2 subunit. The apo β_2 subunit is protected from phenylglyoxal inactivation much more effectively by phosphopyridoxyl-L-serine than by either pyridoxal phosphate or pyridoxine phosphate, both of which lack the L-serine moiety. The phenylglyoxal-modified apo β_2 subunit binds pyridoxal phosphate and the α subunit but cannot bind L-serine or L-tryptophan. We conclude that the α-carboxyl group of L-serine and not the phosphate of pyridoxal phosphate binds to the essential arginyl residue in the β_2 subunit. The specific arginyl residue in the β_2 subunit which is protected by L-serine from modification by phenyl[2-^{14}C]glyoxal has been identified as arginine-148 by isolating a labeled cyanogen bromide fragment (residues 135–149) and by digesting this fragment with pepsin to yield the labeled dipeptide arginine-methionine (residues 148–149). The primary sequence near arginine-148 contains three other basic residues (lysine-137, arginine-141, and arginine-150) which may facilitate anion binding and increase the reactivity of arginine-148. The conservation of the arginine residues 141, 148, and 150 in the sequences of tryptophan synthase from *E. coli*, *Salmonella typhimurium*, and yeast supports a functional role for these three residues in anion binding. The location and role of the active-site arginyl residues in the β_2 subunit and in two other enzymes which contain pyridoxal phosphate, aspartate aminotransferase and glycogen phosphorylase, are compared.

Studies in our laboratory are aimed at identifying functional residues in the active sites of the α and β_2 subunits of tryptophan synthase of *Escherichia coli* (EC 4.1.2.20) in order to understand the mechanism of action of the separate subunits and of the $\alpha_2\beta_2$ complex. [For reviews of tryptophan synthase, see Yanofsky & Crawford (1972) and Miles (1979).] The α subunit catalyzes the conversion of indole-3-glycerol phosphate to indole and D-glyceraldehyde 3-phosphate (reaction 1); this activity is greatly stimulated by the β_2 subunit. The β_2 subunit catalyzes several pyridoxal phosphate dependent reactions, including the synthesis of L-tryptophan from indole and L-serine (reaction 2) and the deamination of L-serine (reaction 4). The α subunit stimulates reaction 2 and inhibits reaction 4. Reaction 3, the physiologically important reaction, is catalyzed only by the $\alpha_2\beta_2$ complex. Several active-site

indole-3-glycerol phosphate $\rightleftarrows$ indole + D-glyceraldehyde 3-phosphate (1)

indole + L-serine $\rightarrow$ L-tryptophan + H_2O (2)

indole-3-glycerol phosphate + L-serine $\rightarrow$ L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O (3)

L-serine $\rightarrow$ pyruvate + ammonia (4)

residues have previously been identified and located in the primary sequence of the β chain. The lysine which forms a Schiff base with pyridoxal phosphate is Lys-87. A histidine which removes the α-proton of L-serine is either His-82 or His-86 (Higgins et al., 1980; Crawford et al., 1980). Cys-230 is the essential cysteine which is protected from chemical modification by the presence of pyridoxal phosphate (Miles & Higgins, 1980).

In the present study, we have investigated the role of arginyl residues in the β_2 subunit of tryptophan synthase by using chemical modification with the arginine-specific reagent phenylglyoxal (Takahashi, 1968, 1977a,b). Studies with phenylglyoxal and similar reagents containing vicinal carbonyl groups (butanedione and cyclohexanedione) have shown that arginyl residues in many proteins bind anionic ligands, including substrates and cofactors, and are unusually reactive with dicarbonyl reagents (Riordan et al., 1977; Patthy & Thész, 1980). Enzymes which have been selectively modified with dicarbonyl reagents include mitochondrial ATPase (Marcus et al., 1976), carboxypeptidase B (Werber et al.,

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1975), alcohol dehydrogenase (Lange et al., 1974), and creatine kinase (Borders & Riordan, 1975). A number of pyridoxal phosphate enzymes have also been studied with dicarbonyl reagents. In some of these enzymes, the phosphate of the coenzyme binds to a specific arginyl residue; these enzymes include γ-aminobutyrate aminotransferase (Tunnicliff, 1980), glutamate decarboxylase (Cheung & Fonda, 1979), and D-serine dehydratase (Kazarinoff & Snell, 1976). In other pyridoxal phosphate enzymes, the α- or distal carboxyl group of the substrate binds to a specific arginyl residue; these enzymes include aspartate aminotransferase (Riordan & Scandurra, 1975; Gilbert & O'Leary, 1975; Miyawaki et al., 1982; Sandmeier & Christen, 1982) and tryptophanase (Kazarinoff & Snell, 1977). Our results show clearly that the β_2 subunit of tryptophan synthase belongs to this second type of pyridoxal phosphate enzymes which require a specific arginyl residue for binding a carboxyl group of the substrate.

The functional arginyl residue has been located in the sequence of only two enzymes which contain pyridoxal phosphate: glycogen phosphorylase (Vandenbunder et al., 1981) and aspartate aminotransferase (Sandmeier & Christen, 1982). In the present study, we have located in the sequence of the β_2 subunit the arginyl residue which binds the α-carboxyl group of L-serine. We have compared the sequence near this residue with the corresponding sequences of the β_2 subunits from *Salmonella typhimurium* and from yeast and with sequences near the functional arginyl residues in glycogen phosphorylase and aspartate aminotransferase.

Materials and Methods

Materials. Phenylglyoxal monohydrate (Aldrich), phenyl[2-^{14}C]glyoxal (32 mCi/mmol, Research Products International Corp.), L-[U-^{14}C]serine (156 mCi/mmol) and Aquasol (New England Nuclear), pyridoxal phosphate (Sigma), pyridoxine phosphate (Mann), and dithiothreitol (Bethesda Research Laboratories), pepsin (Worthington), and Bio-Gel resins (Bio-Rad) were commercial products. Phosphopyridoxyl-L-serine was synthesized as described by Miles et al. (1982). Indole-3-propanol phosphate was a generous gift of Dr. Kasper Kirschner. Indole-3-glycerol phosphate was synthesized enzymatically from indole and fructose 1,6-diphosphate plus aldolase and the $\alpha_2\beta_2$ complex of tryptophan synthase (Hardman & Yanofsky, 1965).

Enzymes and Enzyme Assays. The tryptophan synthase $\alpha_2\beta_2$ complex, β_2 subunit, and α subunit from *E. coli* strain *W3110 trpRcysBΔtrpLD102trpB$^+$trpA$^+$/F'colVBcysB$^+$-ΔtrpLD102trpB$^+$trpA$^+$* were prepared and assayed as described by Higgins et al. (1979). The *E. coli* strain was a generous gift of Dr. C. Yanofsky and of Dr. I. P. Crawford. Protein concentrations were determined by using extinction coefficients reported by Adachi et al. (1974); the protein concentration of modified proteins was determined by the method of Lowry et al. (1951) by using the unmodified protein as a standard. The assay mixture for reaction 2 also contained 0.05 M sodium borate where indicated. The serine deaminase activity (reaction 4) of the β_2 subunit was assayed according to Crawford & Ito (1964). Reaction 1 was measured by a spectrophotometric assay coupled with glyceraldehyde-3-phosphate dehydrogenase as described by Creighton & Yanofsky (1966). The K_m for L-serine and the K_I for phenylglyoxal were determined by using the assays for reactions 2 and 4 modified to contain final concentrations of L-serine from 0.01 to 10 mM and final concentrations of phenylglyoxal from 0.3 to 1.5 mM. The apparent K_m for pyridoxal phosphate was determined by using the assay for reaction 2 modified to contain final concentrations of pyridoxal phosphate from 0.04 to 0.4 μM. For spectrophotometric titrations with pyridoxal phosphate, β_2 subunit (30 μM in monomer) in 1.0 mL of buffer A (see below) was treated sequentially with 11 5-μL aliquots of 1.0 mM pyridoxal phosphate to give final concentrations of 5–52 μM coenzyme. The absorbance values at 430 nm of the solution of enzyme and of a similarly treated control solution without enzyme were determined after each addition and were plotted against the molar ratio of pyridoxal phosphate to β monomer.

Buffers. Buffer A is 50 mM sodium phosphate (pH 7.9) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol. Buffer B is 100 mM sodium borate (pH 7.9) containing 1 mM EDTA and 1 mM dithiothreitol.

Modification of Arginyl Residues with Phenylglyoxal. Solutions of phenylglyoxal were prepared by dissolving the solid in a minimal volume of methanol and then adding water to give a 20 mM solution; the concentration of phenylglyoxal was determined from the absorbance in methanol (ϵ_{247nm} = 11 300 M^{-1} cm^{-1}; Kohlbrenner & Cross, 1978). The extent of arginine modification by phenylglyoxal in phosphate buffer (buffer A) was determined from the difference absorbance at 250 nm, by using the estimated molar extinction coefficient for the diphenylglyoxal adduct of 11 000 M^{-1} cm^{-1} (Takahashi, 1977b). The molar extinction coefficient for the β monomer at 250 nm is 9000 M^{-1} cm^{-1}. Apo β_2 subunit (4.7 mg/mL in buffer A) was incubated for 1 h at 30 °C with 0.1–2.0 mM phenylglyoxal. Reactions were terminated by addition of L-arginine to a final concentration of 10 mM; solutions were dialyzed for 16 h against buffer A in a microdialysis system (Bethesda Research Laboratories) and were diluted 5-fold with buffer A. Solutions were assayed for activity in reaction 2, for absorbance at 250 nm, and for protein (Lowry et al., 1951). Modification by phenylglyoxal in the presence of sodium borate was usually carried out in buffer B at 30 °C by using 2 mM phenylglyoxal for 1 h. When incorporation of radioactivity into protein incubated with various concentrations of phenyl[2-^{14}C]glyoxal was determined, protein solutions were treated for 15 min with 10 volumes of acetone–1 N HCl (39:1 v/v) at 0 °C; the precipitated protein was collected by centrifugation in a Beckman Microfuge A (Sela et al., 1959). Precipitates were dissolved in 2 times the initial volume of 2% Na_2CO_3 in 0.1 N NaOH; aliquots were used for protein determination by the method of Lowry et al. (1951), and other aliquots were counted in 10 mL of Aquasol (New England Nuclear) by using a Beckman LS-345 scintillation counter.

Measurement of the Binding of L-[^{14}C]*Serine by the Method of Hummel & Dreyer (1962).* Each lyophilized sample of control or phenylglyoxal-modified holo $\alpha_2\beta_2$ complex of known amount (26–60 nmol) was dissolved in 0.2 mL of 0.1 M potassium phosphate buffer, pH 7.8, containing 5 mM EDTA, 0.2 mM dithiothreitol, 0.02 mM pyridoxal phosphate, and 0.03, 0.06, 0.10, or 0.15 mM L-[^{14}C]serine (0.167 μCi/mol) and was applied to a Sephadex G-25 (fine) column (0.8 cm × 30 cm) which had been equilibrated at 4 °C with 200 mL of the buffer containing L-[^{14}C]serine. Fractions (12 drops = 0.515 mL) were collected, and 0.1-mL aliquots were counted in Aquasol after addition of 0.1 mL of 1 N acetic acid. The number of nanomoles of L-serine bound was calculated from the difference between the number of counts in the base line and the number of counts in the trough corrected for the volume of the aliquot counted and for the specific activity. The binding ratio r (=moles of L-serine bound per mole of enzyme) is calculated from these data and plotted vs. $r/[L]$ where [L] is the free ligand concentration. The straight lines drawn through each set of data points have a slope of $-K_d$ and an

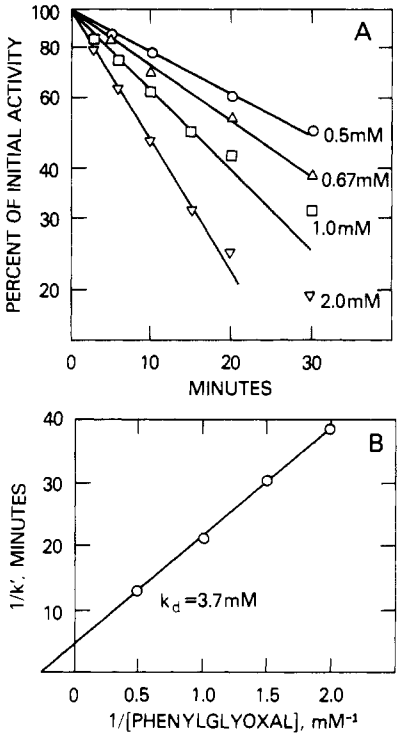

FIGURE 1: Effect of phenylglyoxal concentration upon the rate of inactivation of the apo β_2 subunit of tryptophan synthase. (A) Apo β_2 subunit (0.75 mg/mL in buffer B) was incubated with the indicated concentrations of phenylglyoxal at 30 °C: 0.5 mM (○); 0.67 mM (△); 1.0 mM (□); 2.0 mM (▽). Aliquots (0.005 mL) were removed at intervals for assay of reaction 2 in the presence of excess α subunit and 0.05 M sodium borate. (B) Reciprocal first-order rate constants (k') calculated from (A) are plotted vs. the reciprocal concentration of phenylglyoxal. The dissociation constant (K_d) for phenylglyoxal calculated from the intercept on the abscissa ($1/K_d$) is 3.7 mM.

abscissa intercept of n where K_d is the binding constant and n is the number of moles of ligand bound per mole of enzyme at an infinite concentration of ligand.

Spectrophotometric Titration of the $\alpha_2\beta_2$ Complex with Amino Acids. The absorbance of solutions of control or modified holo $\alpha_2\beta_2$ complex (0.01 mM in $\alpha\beta$ monomer in buffer A or in buffer A minus dithiothreitol for titrations with L-serine) at 410, 476, or 460 nm was determined by using a Cary 118 spectrophotometer at 23 °C after each addition of L-serine, L-tryptophan, or D-tryptophan, respectively; the observed absorbance was corrected for changes in volume and for the initial absorbance of the solution before addition of amino acid. The maximum absorbance change ($\Delta\epsilon_{max}$) was estimated from a plot of $1/\Delta\epsilon$ vs. $1/S_T$ where S_T is the total ligand concentration; the straight line through the data points at high S_T intersects the y axis at $1/\Delta\epsilon_{max}$. The experimental data are then plotted as log $[R/(1-R)]$ vs. log C_{free} where $R = \Delta\epsilon/\Delta\epsilon_{max}$ and $C_{free} = S_T - (RE_T)$ where E_T is the enzyme monomer concentration (0.01 mM).

Cyanogen Bromide Treatment and Fractionation. Lyophilized protein was dissolved at 10 mg/mL in 70% formic acid, treated with excess cyanogen bromide for 24 h at 23 °C, diluted 10-fold with water, and lyophilized. The lyophilized digest was dissolved in 0.5–1.0 mL of 9% formic acid and fractionated by gel filtration on a 1.5 cm × 110 cm or 1.5 cm × 180 cm column on Bio-Gel P-10 in 9% formic acid, collecting 1.5-mL fractions.

Amino Acid Analysis. Peptides (1–10 nmol) were hydrolyzed in vacuo in 6 N HCl (Pierce, 0.5 mL) at 110 °C for 24 h. Hydrolysates were analyzed on a Beckman 121 M amino acid analyzer by using the single-column methodology. In some cases, the temperature of the elution was changed from 50 to 45.6 °C to permit separation of homoserine from glutamate.[1]

Table 1: Effect of L-Serine, Pyridoxal Phosphate, and Pyridoxal Phosphate Analogues upon the Rate of Inactivation of the Apo β_2 Subunit by Phenylglyoxal[a]

addition	k' (min^{-1})
none	0.033
0.1 M L-serine	0.029
0.2 mM pyridoxal phosphate	0.022
0.2 mM pyridoxal phosphate + 0.1 M L-serine	0.004
2.0 mM phosphopyridoxyl-L-serine	0.009
2.0 mM pyridoxine phosphate	0.046

[a] Apo β_2 subunit (0.62 mg/mL in buffer B) was incubated at 29 °C with 2 mM phenylglyoxal in the presence of the indicated additions and assayed at intervals as in Figure 1A. First-order rate constants (k', min^{-1}) were calculated from plots of the data as in Figure 1A.

Results

Kinetics of Inactivation of the Apo β_2 Subunit by Phenylglyoxal. The rate of inactivation of the apo β_2 subunit by excess phenylglyoxal follows pseudo-first-order kinetics (Figure 1A). A plot of the reciprocal first-order rate constants vs. the reciprocal concentration of phenylglyoxal (Figure 1B) gives a dissociation constant for phenylglyoxal (K_d = 3.7 mM). The slope of a plot of the log of the apparent first-order rate constants for inactivation from Figure 1A vs. the log of the concentration of the inactivator is 0.8, the number of molecules of the inactivator reacting per enzyme active site (Levy et al., 1963) (data not shown). Although 2,3-butanedione, another arginine-specific dicarbonyl reagent, and phenylglyoxal both inactivate the apo β_2 subunit at the same rate (data not shown), phenylglyoxal has been used here since it gives a more stable adduct (Takahashi, 1968, 1977a,b) and is available in radioactive form.

Protection of the β_2 Subunit by L-Serine and by Phosphopyridoxyl-L-serine. The apo β_2 subunit is rapidly inactivated by 2 mM phenylglyoxal both in the absence and in the presence of 0.1 M L-serine (Table I). Whereas pyridoxal phosphate alone only weakly protects the apo β_2 subunit from inactivation, pyridoxal phosphate and L-serine together strongly protect the apo β_2 subunit. Phosphopyridoxyl-L-serine strongly protects the apo β_2 subunit, whereas pyridoxine phosphate offers no protection and even enhances the rate of inactivation somewhat. Since these two pyridoxal phosphate analogues bind to the apo β_2 subunit with nearly identical affinities (Tschopp & Kirschner, 1980), the L-serine part of the pyridoxal phosphate analogue is the essential part in preventing inactivation.

Protection of the $\alpha_2\beta_2$ Complex by Substrates and Substrate Analogues. Addition of L-serine, L-tryptophan, or D-tryptophan to the holo $\alpha_2\beta_2$ complex results in the formation of stable enzyme–substrate intermediates which can be observed spectrophotometrically (Miles, 1980). These three amino acids significantly protect the holo $\alpha_2\beta_2$ complex but not the apo $\alpha_2\beta_2$ complex from inactivation and from modification (Table II). The nonreactive L-alanine shows no protection. Indole-3-glycerol phosphate (a substrate of the α subunit) and indole-3-propanol phosphate (an analogue of indole-3-glycerol phosphate) both partially protect the apoenzyme but not the

[1] We are grateful to Barbara F. Torain, Laboratory of Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, for the amino acid analysis.

Table II: Effect of Pyridoxal Phosphate and of Substrates and Analogues upon Modification and Inactivation of the $\alpha_2\beta_2$ Complex by Phenyl[2-^{14}C]glyoxal[a]

	apo $\alpha_2\beta_2$ (−pyridoxal phosphate)				holo $\alpha_2\beta_2$ (+pyridoxal phosphate)			
addition	fraction of initial activity	fraction of activity protected[c]	mol of Arg modified[d]/ mol of $\alpha\beta$	mol of Arg protected[e]	fraction of initial activity	fraction of activity protected[c]	mol of Arg modified[d]/ mol of $\alpha\beta$	mol of Arg protected[e]
none (control)	0.46	[0]	2.5	[0]	0.43	[0]	2.1	[0]
20 mM L-Ser	0.45	0	2.6	0	0.90	0.82	0.8	1.3
20 mM L-Ala	0.44	0	2.4	0.1	0.47	0.07	1.9	0.2
0.4 mM IPP[b]	0.64	0.33	1.7	0.8	0.41	0	1.6	0.5
2 mM IGP[b]	1.13	1.2	0.9	1.6	0.56	0.23	1.6	0.5
4 mM L-Trp	0.44	0	2.2	0.3	0.71	0.49	1.2	0.9
4 mM D-Trp	0.43	0	2.4	0.1	0.87	0.77	1.0	1.1

[a] Apo $\alpha_2\beta_2$ complex (0.034 mM) was incubated in the presence or absence of 0.5 mM pyridoxal phosphate in buffer B for 45 min at 30 °C with 2 mM phenyl[2-^{14}C]glyoxal (920 cpm/nmol). Substrates and analogues were added at the indicated concentrations. Aliquots were diluted 10-fold in buffer B containing 5 mM L-arginine and 1.0 mM pyridoxal phosphate before assay in reaction 2 in the presence of 0.05 M sodium borate. Incorporation of radioactivity was determined as described under Materials and Methods. [b] Abbreviations: IPP, indole-3-propanol phosphate; IGP, indole-3-glycerol phosphate. [c] (Fraction of initial activity − fraction of initial activity of control)/(1.0 − fraction of initial activity of control). [d] The label incorporated into the apo or holo $\alpha_2\beta_2$ complex (2.5 or 2.1 mol/mol of $\alpha\beta$, respectively) is distributed about equally between the α and β_2 subunits. Treatment of the α subunit alone with 2 mM phenylglyoxal does result in modification but has little effect on the activity of the α subunit in reaction 2 (unpublished results). [e] Moles of arginine modified per mole of $\alpha\beta$ with no addition (control) − moles of arginine modified per mole of $\alpha\beta$ with indicated addition.

holoenzyme from inactivation and from modification. Since treatment of the α subunit alone under the same conditions causes no decrease in the ability of the α subunit to stimulate the tryptophan synthase activity (reaction 2) of the β_2 subunit (data not shown), the observed protective effects of substrates upon the activity of the $\alpha_2\beta_2$ complex are due to effects upon the β_2 subunit. When the holo $\alpha_2\beta_2$ complex is modified with phenyl[2-^{14}C]glyoxal, the label is distributed about equally between the α and β_2 subunits (Table II, footnote d, and Figure 3). Thus, residues in the α subunit which are not essential for stimulation of the β_2 activity are modified by phenylglyoxal; the possibility that one or more of these residues is essential for the activity of the α subunit in reaction 1 is under current investigation.

Competition between Phenylglyoxal and L-*Serine for Binding.* Phenylglyoxal is a competitive inhibitor of L-serine in the assay of reaction 2 with the $\alpha_2\beta_2$ complex and in the assay of reaction 4 with the β_2 subunit [see Materials and Methods and Figure 1A,B of the supplementary materials (see paragraph at end of paper regarding supplementary material)]. Inhibition by phenylglyoxal results from reversible binding rather than from irreversible inactivation since linear initial rates were observed. Phenylglyoxal is noncompetitive with indole (data not shown). Thus, phenylglyoxal and L-serine compete for the same binding site, presumably an arginyl residue. The calculated inhibition constants (K_I) for phenylglyoxal are 0.3 mM with the holo $\alpha_2\beta_2$ complex and 0.9 mM with the holo β_2 dimer. The finding that the K_I for phenylglyoxal for the holo β_2 dimer is 4-fold lower than the K_d for apo β_2 determined in Figure 1B is probably due to effects of pyridoxal phosphate and possibly of L-serine on the conformation of the β_2 subunit which make the holo β_2 subunit bind phenylglyoxal more tightly.

Phenylglyoxal Modification Prevents L-*Serine Binding.* Using the gel filtration method of Hummel & Dreyer (1962) as described by Ackers (1975), we have measured the binding of L-[^{14}C]serine to the holo $\alpha_2\beta_2$ complex, to complex modified with 2 mM phenylglyoxal in the presence or absence of L-serine, and to complex modified with 10 mM phenylglyoxal (Table III and Figure 2A,B of the supplementary material). The finding that the amount of L-serine bound is equal to the fraction of the active enzyme means that only the active fraction of the holo $\alpha_2\beta_2$ complex binds L-serine. Thus, the residual activity observed in partially inactivated enzyme preparations is due to a fraction of the enzyme which has not been modified. This conclusion is consistent with the finding that the K_m for L-serine in reaction 2 is essentially unchanged by modification of either the apo β_2 subunit (K_m = 0.5 mM before modification and 0.8 mM after modification) or the holo $\alpha_2\beta_2$ complex (K_m = 0.8 mM before modification and 0.6 mM after modification) (see Materials and Methods).

Table III: Effect of Phenylglyoxal Modification of the Holo $\alpha_2\beta_2$ Complex upon L-Serine Binding

addition during modification[a]	fraction of activity remaining[a]	mol of L-serine bound per mol of $\alpha\beta$, n[b]	K_d[b] (μM)
none	[1.0]	0.96	39
2 mM phenylglyoxal	0.59	0.53	40
10 mM phenylglyoxal	0.24	0.26	45
2 mM phenylglyoxal + 20 mM L-serine	0.98	0.96	39

[a] Holo $\alpha_2\beta_2$ complex (0.14 mM) in 0.1 M sodium borate buffer, pH 7.9, containing 1 mM EDTA was incubated for 1 h at 30 °C with the indicated additions and then assayed for activity in reaction 2. [b] Binding of L-serine was determined by gel filtration of each of the four enzyme preparations on a column equilibrated with 0.03, 0.06, 0.10, or 0.15 mM L-[^{14}C]serine (see Materials and Methods and Figure 2 of the supplementary material). When the binding ratio r (=moles of L-serine bound per mole of enzyme) is plotted vs. r/[L] where [L] is the free ligand concentration, a straight line with a slope of $-K_d$ and an abscissa intercept of n is drawn through each set of data points (Figure 2 in the supplementary material). K_d is the binding constant, and n is the number of moles of ligand bound per mole of enzyme ($\alpha\beta$) at an infinite concentration of the ligand.

Spectral Properties of Modified Holo $\alpha_2\beta_2$ Complex in the Presence of Substrates. The spectral changes observed upon addition of L-serine, L-tryptophan, or D-tryptophan to the holo $\alpha_2\beta_2$ complex (Miles et al., 1968; Goldberg et al., 1968; Miles, 1980; Lane & Kirschner, 1981) can be used for spectrophotometric titrations. Table IV summarizes the results of spectrophotometric titrations shown in Figure 3 of the supplementary material. Since the absorbance changes observed upon titration of the phenylglyoxal-modified holo $\alpha_2\beta_2$ complex (33% residual activity) with L-serine and L-tryptophan are exactly 33% of the absorbance changes observed with the control, we conclude that the 67% of the enzyme which is modified cannot bind L-serine or L-tryptophan at all. This

Table IV: Effect of Phenylglyoxal Modification upon the Spectrophotometric Titration of the Holo $\alpha_2\beta_2$ Complex with Amino Acids[a]

substrate	λ_{max} (nm)	$\Delta\epsilon_{max}$ [(mol of subunit)$^{-1}$ cm^{-1}]			$[S]_{0.5}$ (mM)	
		control	modified	modified − (0.33)control	control	modified − (0.33)control
L-serine	410	−3900	−1300	0	0.009	no binding
L-tryptophan	476	2400	800	0	0.14	no binding
D-tryptophan	460	4300	2400	1000	0.03	0.3

[a] Spectrophotometric titrations were carried out as described under Materials and Methods by using modified holo $\alpha_2\beta_2$ complex (treated at 23 mg/mL with 5 mM phenylglyoxal in buffer B for 2 h at 30 °C followed by dialysis against buffer B containing 0.02 mM pyridoxal phosphate for 48 h at 4 °C and then against buffer A for 24 h at 4 °C) which had 33% of the activity of an untreated control which was dialyzed under the same conditions. Hill plots of spectral titration data are shown in Figure 3 of the supplementary material. Since the modified $\alpha_2\beta_2$ complex had 33% residual activity, absorbance changes were corrected for the calculated binding by 33% unmodified enzyme. The half-saturation values ($[S]_{0.5}$) are obtained from the Hill plots where log $[R/(1-R)] = 0$.

confirms the direct binding studies with L-[^{14}C]serine (Table III). In contrast, D-tryptophan is bound to the modified enzyme since the absorbance changes observed with modified enzyme are significantly larger than those expected for the 33% fraction which is not modified (Table IV). The half-saturation value, $[S]_{0.5}$, for binding of D-tryptophan to the modified enzyme (corrected for the unmodified fraction) is 0.3 mM, 10 times higher than $[S]_{0.5}$ for the control (0.03 mM) (Table IV).

Modified β_2 Subunit Forms a Stoichiometric $\alpha_2\beta_2$ Complex. The amount of α subunit which binds to the β_2 subunit modified with 0.8 mM phenylglyoxal for 0, 15, 30, or 60 min is determined by titrating the β_2 subunit with α subunit and measuring the activity of each mixture in reaction 2. The results show that about 0.9 mol of α binds to 1.0 mol of β monomer in each preparation. Thus, the modified β_2 subunit forms a stoichiometric $\alpha_2\beta_2$ complex which has reduced activity in reaction 2.

Modified β_2 Subunit and $\alpha_2\beta_2$ Complex Bind Pyridoxal Phosphate. The spectrophotometric titration of phenylglyoxal-modified apo β_2 subunit (22% residual activity) with pyridoxal phosphate shows that 0.85 mol of pyridoxal phosphate is bound per mol of β monomer (see Materials and Methods). The apparent K_m for pyridoxal phosphate measured in reaction 2 in the presence of excess α subunit is 0.07 μM for untreated β_2 subunit and 0.2 μM for phenylglyoxal-modified β_2 subunit with 22% residual activity (see Materials and Methods). These results indicate that pyridoxal phosphate is bound to phenylglyoxal-modified apo β_2 subunit in a slightly altered way. In contrast, modification of the holo $\alpha_2\beta_2$ complex (33% residual activity) results in essentially no change in the absorption spectrum of the bound pyridoxal phosphate (spectrum of enzyme used in Table IV).

Inactivation by Phenylglyoxal Results from Modification of an Arginyl Residue. The extent of modification of the apo β_2 subunit by phenylglyoxal has been determined by two methods (Figure 2). In the first method, a diphenylglyoxal derivative of arginine which is formed in phosphate buffer (buffer A) is quantitated from its absorbance at 250 nm [see inset of Figure 2, Materials and Methods, and Takahashi (1977b)]. The second method measures incorporation of radioactivity from phenyl[2-^{14}C]glyoxal in borate buffer (buffer B). Under these conditions, a monophenylglyoxal derivative which is more stable than the diphenylglyoxal derivative but which has no characteristic absorbance is formed (Borders & Riordan, 1975). When the extent of modification determined by each of these methods is plotted vs. the percent activity remaining (Figure 2), the two sets of points are similar under conditions resulting in the loss of less than 50% activity. Extrapolation of the line from the data above 50% residual activity shows that one arginyl residue must be modified for

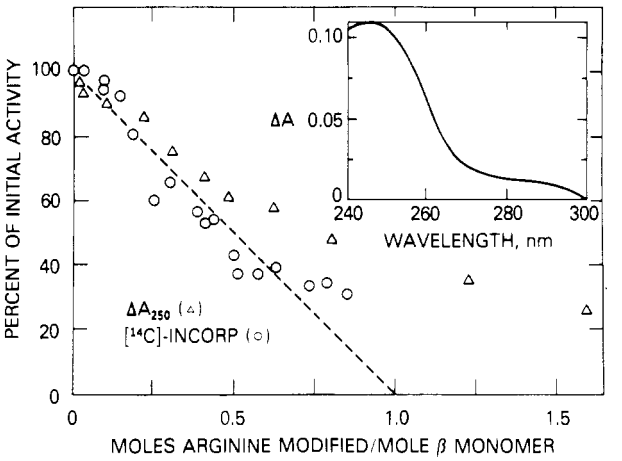

FIGURE 2: Effect of the extent of modification of the apo β_2 subunit by phenylglyoxal upon activity. The extent of modification of arginyl residues determined from the change in absorbance at 250 nm after modification by phenylglyoxal in phosphate buffer (buffer A) (△) or from the incorporation of radioactivity after modification by phenyl[2-^{14}C]glyoxal in borate buffer (buffer B) (○) (see Materials and Methods) is plotted vs. the percent of initial activity. The inset shows the difference absorbance spectrum between 10.5 μM apo β_2 subunit modified with 1 mM phenylglyoxal in buffer A (0.8 mol of arginine modified per mol of β monomer) and unmodified enzyme. The absorbance of the unmodified enyzme was 0.105 at 250 nm and 0.27 at 278 nm.

total inactivation, suggesting that one arginyl residue is important for activity. However, as complete inactivation is approached, more extensive labeling of the enzyme is observed.

Amino acid analysis of an acid hydrolysate of apo β_2 subunit modified in phosphate buffer (1.6 mol of arginine modified/mol of monomer) shows the disappearance of about 1 mol of arginine; the amount of histidine and lysine does not change significantly (data not shown). The latter two amino acids may react with phenylglyoxal under some conditions (Takahashi, 1968, 1977a). The partial regeneration of arginine during acid hydrolysis has been reported (Vandenbunder et al., 1981). The sulfhydryl content determined with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 0.1% sodium dodecyl sulfate (Ellman, 1959) is unchanged by modification (data not shown). Since butanedione has been reported to produce photodestruction of several amino acids in *Aeromonas* aminopeptidase (Mäkinen et al., 1982), we compared the inactivation of the apo β_2 subunit by 2 mM phenylglyoxal in borate buffer in the light, in the dark, and in the presence of 2 mM sodium azide, a scavenger of singlet oxygen. Since the extent of inactivation after 60 min is the same in each case, we conclude that inactivation by phenylglyoxal is not due to photochemical inactivation.

Isolation and Identification of Peptides Containing an Arginyl Residue Protected by L-Serine. The presence of L-

Table V: Effect of L-Serine upon Modification by Phenyl[2-^{14}C]glyoxal: Isolation of Protected Peptides[a]

sample	enzyme	additions	residual activity (%)	incorpn (%) total	incorpn (%) CB V
1	holo $\alpha_2\beta_2$	none	20	[100]	[100]
2	holo $\alpha_2\beta_2$	20 mM L-serine	92	25	10
3	holo β_2	none	20	[100]	[100]
4	holo β_2	0.5 M L-serine[b]	80	22	4

[a] Holo $\alpha_2\beta_2$ complex (samples 1 and 2) (2.9 mg) was modified in the presence or absence of 20 mM L-serine with 2 mM phenyl-[2-^{14}C]glyoxal (6340 cpm/nmol) in buffer B for 1 h at 30 °C; residual activity and total incorporation of radioactivity into $\alpha_2\beta_2$ complex were determined (435 × 10^3 cpm were incorporated into sample 1 = 100%). Protein precipitated with acetone–HCl (Sela et al., 1959) was treated with cyanogen bromide and fractionated on Bio-Gel P-10 as described under Materials and Methods (Figure 3A). Fractions in the major radioactive peak (fraction 165–181), designated CB V, were combined and analyzed for radioactivity (80 × 10^3 cpm in sample 1 = 100%). Holo β_2 subunit (samples 3 and 4) (1.8 mg) was modified in the presence and absence of 0.5 M L-serine with 2 mM phenyl[2-^{14}C]-glyoxal (6690 cpm/nmol) in buffer B for 40 min at 30 °C; residual activity and total incorporation of radioactivity into β_2 subunit were determined (211 × 10^3 cpm were incorporated into sample 3 = 100%). Fractionation of the cyanogen bromide digest as described above and in Figure 3B yielded a radioactive peak (fraction 115–125), designated CB V, from sample 3 containing 79 × 10^3 cpm. [b] This high concentration of L-serine was used since L-serine is converted to pyruvate and ammonia by the holo β_2 subunit but not by the holo $\alpha_2\beta_2$ complex.

serine greatly reduces the total incorporation of radioactivity from phenyl[2-^{14}C]glyoxal into either the holo $\alpha_2\beta_2$ complex or the holo β_2 subunit and largely protects both enzymes from inactivation (Table V). Gel filtration of cyanogen bromide fragments obtained from phenyl[2-^{14}C]glyoxal-modified holo $\alpha_2\beta_2$ complex (Figure 3A) and holo β_2 subunit (Figure 3B) shows the presence of major radioactive peaks at fractions 165–181 (Figure 3A) or fractions 115–125 (Figure 3B) which comigrate (Figure 3B). The peptide fragment in these peaks was identified as CB V (residues 135–149) from its amino acid composition (Table VI); CB V contains two arginyl residues at positions 141 and 148 (Crawford et al., 1980). Further digestion of CB V by pepsin, followed by gel filtration on Bio-Gel P-2, yielded a major radioactive peak which was identified from its amino acid composition as residues 148–149 (Table VI). CB V was strongly protected from modification in sample 2 (from holo $\alpha_2\beta_2$ plus L-serine) and in sample 4 (from holo β_2 plus L-serine) (Table V). The other prominent radioactive peak in Figure 3A (fractions 85–100) eluted at a position expected for the large cyanogen bromide fragment of the α subunit CB III (residues 102–251) which contains six arginine residues (Li & Yanofsky, 1972) and is not seen in Figure 3B. Thus, the arginine of the β_2 subunit which is protected by L-serine in the holo β_2 subunit and in the holo $\alpha_2\beta_2$ complex is Arg-148.

Discussion

The irreversible inactivation of the β_2 subunit by phenylglyoxal (Figure 1A) appears to follow initial reversible binding of phenylglyoxal to the β_2 subunit since inactivation by phenylglyoxal shows saturation kinetics (Figure 1B). Since phenylglyoxal is a competitive inhibitor of L-serine, phenyl-

Table VI: Amino Acid Composition of Isolated Peptides and Expected Sequences

amino acid	CNBr fragment[a] nmol	CNBr fragment[a] residues/mol[b]	CB V (135–149)[c] (residues/mol)	pepsin peptide[d] nmol	pepsin peptide[d] residues/mol[e]	148–149[c] (residues/mol)
Asp	6.6	2.1	2	0.4		
Thr				0.2		
Ser	2.6	0.8	1	0.4		
Glu	7.1	2.2	2	0.1		
Pro	3.6	1.1	1			
Gly	3.7	1.1	1	0.7		
Ala	3.7	1.1	1			
Cys						
Val	7.2	2.2	2			
Met (Hse)	2.0	0.6	1	3.3	1.0	1
Ile						
Leu				0.5		
Tyr						
Phe	3.0	0.9	1			
Lys	3.2	1.0	1			
His						
Arg	5.6 48.2[f]	1.7 15[f]	2 15[f]	3.4 6.7[e,f]	1.0 2[f]	1 2[f]
radioactivity	3.4	1.0		5.0	1.5	

[a] This fragment was isolated from phenyl[2-^{14}C]glyoxal-modified apo β_2 subunit by cyanogen bromide digestion and fractionation on Bio-Gel P-10 as described under Materials and Methods and in Table V and Figure 3. The fragment was purified further by fractionation on Bio-Gel P-4 before acid hydrolysis and amino acid analysis at 45.6 °C to detect homoserine. The material submitted to acid hydrolysis and to amino acid analysis contained 3.4 nmol of radioactive label (=12 700 cpm ÷ 3760 cpm/nmol). [b] The number of residues per mole is calculated from the data [assuming that the peptide is CB V (see footnote *c*)] by the relationship residues/mol = [nmol of amino acid × number of residues in peptide (15)]/[total nmol of amino acid found in peptide (48.2)]. [c] Calculated compositions for peptides shown from the sequence of the β_2 subunit as determined by DNA sequencing (Crawford et al., 1980). [d] The lyophilized CNBr fragment containing 30 nmol of radioactive label (=118 × 10^3 cpm ÷ 3760 cpm/nmol) was dissolved in 0.1 mL of 5% formic acid and treated with 0.8 nmol of pepsin for 3 h at 23 °C. Pepsin (0.8 nmol) was again added 1 h after the digestion was started. The digest was fractionated on a Bio-Gel P-2 column (0.8 cm × 110 cm) in 9% formic acid, and 1-mL fractions were collected. Two-thirds of the radioactivity recovered from the column eluted in fractions 81–90 (72 × 10^3 cpm). An aliquot of the peak fractions which contained 5.0 nmol of radioactive label (=19 000 cpm ÷ 3760 cpm/nmol) was acid hydrolyzed, lyophilized, and treated with 0.05 mL of 10:0.4:90 pyridine:acetic acid:water (pH 6.5) at 105 °C for 1 h to convert homoserine lactone to homoserine (Amber, 1965) and dried and subjected to amino acid analysis at 45.6 °C to detect homoserine. [e] The number of residues per mole is calculated from the data (assuming that the peptide contains residues 148–149 and that other amino acids found are impurities) by the relationship residues/mol = [nmol of amino acid × number of residues in peptide (2)]/[total nmol of amino acid found in the peptide (6.7)]. [f] Total.

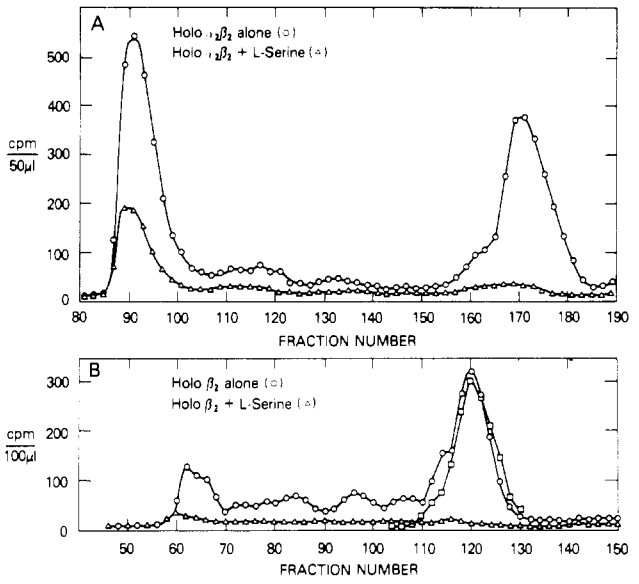

FIGURE 3: Gel filtration on Bio-Gel P-10 of cyanogen bromide fragments obtained from phenyl[2-^{14}C]glyoxal-modified holo $\alpha_2\beta_2$ complex (A) and phenyl[2-^{14}C]glyoxal-modified holo β_2 subunit (B). Enzymes were modified in the presence or absence of L-serine as described in Table V: samples 1 and 2 (A) and samples 3 and 4 (B). Control (enzyme alone) (○); enzymes modified in the presence of L-serine (△); peak fractions 165–175 from (A) reapplied to the column used in (B) (□). The column size was 1.5 cm × 180 cm in (A) and 1.5 cm × 110 cm in (B); elution was with 9% formic acid.

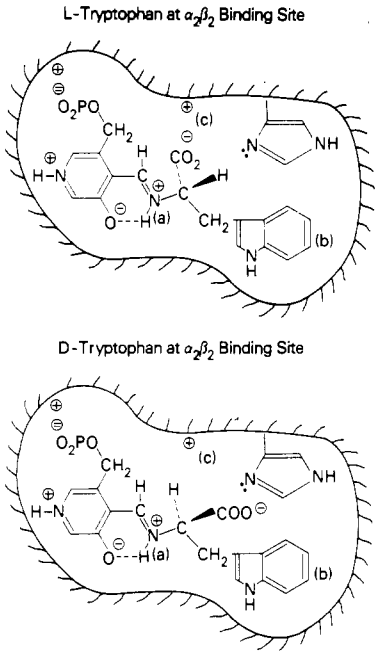

FIGURE 4: Scheme depicting binding of L-tryptophan and D-tryptophan to the active site of the $\alpha_2\beta_2$ complex. The amino group of L- and D-tryptophan binds in a Schiff base linkage with pyridoxal phosphate at site (a); the indolyl moiety of L- and D-tryptophan binds to a hydrophobic site (b); the carboxyl group of L-tryptophan binds to Arg-148 (this work) at site (c) whereas the carboxyl group of D-tryptophan is oriented in the opposite direction. The histidyl residue (His-82 or His-86) catalyzes the removal of the α-proton of L-tryptophan but not of D-tryptophan [see Miles (1980)].

glyoxal and L-serine may both bind to the same site.

The kinetic data indicate that inactivation is due to reaction of about one molecule of inactivator per monomer (n = 0.8) (see Results). This result and the results of studies of phenylglyoxal incorporation (Figure 2) show that inactivation by phenylglyoxal results from modification of a single residue. Evidence that this residue is arginine comes from the difference spectrum (inset of Figure 2), from the amino acid analysis, and from the isolation of a peptide labeled at Arg-148. Although phenylglyoxal can react with more than one arginyl residue per β monomer under conditions resulting in more than 50% inactivation (Figure 2), the results of substrate protection experiments indicate that a single arginyl residue is essential for activity. When either the holo β_2 subunit or the holo $\alpha_2\beta_2$ complex is treated with phenylglyoxal in the presence of L-serine, inactivation is greatly reduced, and the modification of a single arginyl residue (Arg-148) in the β_2 subunit is almost completely prevented (Tables I, II, and V). In addition to L-serine, L-tryptophan and D-tryptophan also protect the holo $\alpha_2\beta_2$ complex from inactivation and from modification by phenylglyoxal (Table II). L-Serine does not protect the apo β_2 subunit and the apo $\alpha_2\beta_2$ complex from phenylglyoxal inactivation since pyridoxal phosphate is required for L-serine binding. Our finding that phosphopyridoxyl-L-serine protects the apo β_2 subunit from inactivation by phenylglyoxal much more effectively than do pyridoxal phosphate and pyridoxine phosphate which lack the L-serine moiety supports the conclusion that the modifiable arginine binds the α-carboxyl group of L-serine and not the phosphate of pyridoxal phosphate.

The role of the modifiable arginyl residue is further defined by comparing the binding properties of the untreated and phenylglyoxal-modified enzymes. These studies have been complicated by the presence of a fraction of the $\alpha_2\beta_2$ complex which has not been modified. Attempts to separate the modified and unmodified fractions by several methods, including gel filtration and ion-exchange chromatography, were unsuccessful. When corrections are made for the unmodified fraction, the modified $\alpha_2\beta_2$ complex is found to be unable to bind L-serine (Table III) and L-tryptophan (Table IV) but to be able to bind D-tryptophan with a 10-fold lowered affinity (Table IV). The inability of the enzyme to bind the L-amino acid substrates is consistent with a role for an arginyl residue in binding the α-carboxyl group of the L-amino acids. Previous studies (Miles, 1980) have suggested that "L-tryptophan may normally bind by three types of interaction: (a) the (pyridoxal phosphate) Schiff base linkage; (b) the interaction of the indolyl moiety with a hydrophobic site; and (c) the electrostatic interaction of the carboxyl group with a positively-charged group. If D-tryptophan binds to sites (a) and (b), its carboxyl group will be oriented in the opposite direction from that of the group at site (c)." This proposal is illustrated in Figure 4 and is supported by our present finding that modification of an arginyl residue at site (c) prevents the binding of L-tryptophan but not the binding of D-tryptophan. The fact that the modified $\alpha_2\beta_2$ complex can still bind D-tryptophan is important evidence that modification has not produced nonspecific steric or conformational changes which prevent substrate binding. The presence of the bulky phenylglyoxal moiety at site (c) may interfere with binding of the D-tryptophan to the nearby sites (a) and (b) and be responsible for the decreased affinity of the modified $\alpha_2\beta_2$ complex for D-tryptophan. As a corollary, bound D-tryptophan may protect the holo $\alpha_2\beta_2$ complex from inactivation (Table II) by preventing access of phenylglyoxal to the active site. D-Tryptophan could act either directly by binding close to the active-site arginine (see Figure 4) or indirectly by making the entire active-site region less accessible to solvent. The latter possibility is supported by our finding that D-tryptophan protects the pyridoxal phosphate in the active site of the holo $\alpha_2\beta_2$ complex from reduction by sodium borohydride (Miles et al., 1982).

The protective effects of indole-3-glycerol phosphate and indole-3-propanol phosphate (Table II) are surprising since

these compounds bind to the active site of the α subunit (Kirschner et al., 1975). Protection might be due to direct interaction with the essential arginyl residue of the β_2 subunit in a composite active site or to indirect effects upon the active site of the β_2 subunit resulting from a change in subunit interaction. The finding that indolepropanol phosphate protects the apo $\alpha_2\beta_2$ complex more effectively than the holo $\alpha_2\beta_2$ complex is also unexplained. Since indirect protective effects may occur in the $\alpha_2\beta_2$ complex, the results of protection experiments with the β_2 subunit are more definitive than those with the $\alpha_2\beta_2$ complex.

We conclude that the primary site of phenylglyoxal modification which affects L-serine binding does not affect pyridoxal phosphate binding, since apo β_2 subunit binds 0.85 mol of pyridoxal phosphate per β monomer as shown by spectrophotometric titration and since the spectrum of enzyme-bound pyridoxal phosphate is not altered in the modified holo $\alpha_2\beta_2$ complex. The changes observed in the apparent K_m for pyridoxal phosphate probably result from phenylglyoxal modification at a secondary site which is less susceptible to modification in the holo $\alpha_2\beta_2$ complex. Since phenylglyoxal modification of the β_2 subunit prevents binding of L-serine and L-tryptophan but does not prevent binding of D-tryptophan, pyridoxal phosphate, or α subunit, we conclude that this modification of the β_2 subunit specifically prevents the binding of the α-carboxyl group of L-serine and L-tryptophan.

The localization of phenylglyoxal-modifiable arginyl residues has not been frequently undertaken since some of the reaction products are unstable and since the chemistry and stoichiometry of the phenylglyoxal–arginine reaction are still not completely characterized (Vandenbunder et al., 1981). Whereas phenylglyoxal is thought to form an unstable 2:1 complex with arginine in the absence of borate buffer (Takahashi, 1968), phenylglyoxal appears to form a more stable 1:1 complex when the initial reaction is carried out in borate buffer (Borders & Riordan, 1975; Werber et al., 1975). We have found that even the more stable 1:1 complex breaks down under weakly alkaline conditions and in organic solvents. Since weak acids stabilize the label, cyanogen bromide digestion, pepsin digestion, and gel filtration were all performed in formic acid. Our finding of 1.0 mol of phenyl[2-^{14}C]glyoxal incorporated per mol of cyanogen bromide fragment CB V (based on amino acid analysis) confirms the 1:1 stoichiometry of the arginine–phenylglyoxal interaction since the results of pepsin digestion show that only one of the two arginyl residues (Arg-148) in this fragment is labeled. The finding of 1.5 mol of phenyl[2-^{14}C]glyoxal incorporated per mol of pepsin peptide could result from low recovery of homoserine and from incomplete regeneration of arginine upon acid hydrolysis.

Cyanogen bromide treatment of phenylglyoxal-modified holo $\alpha_2\beta_2$ complex also produces a high molecular weight fragment, which is not present in the β_2 subunit; this fragment is probably the large cyanogen bromide fragment III of the α subunit (residues 102–251) containing six arginyl residues (Li & Yanofsky, 1972). The significant protection (65%) of this fragment when modification is performed in the presence of L-serine may result from the fact that L-serine increases the association constant for $\alpha_2\beta_2$ complex formation (Creighton & Yanofsky, 1966) and may thus indirectly protect from modification residues which are "buried" in the subunit interaction site. Our finding that the same arginyl residue in the holo β_2 subunit and in the holo $\alpha_2\beta_2$ complex is protected by L-serine from phenylglyoxal modification is evidence that L-serine interacts directly with Arg-148 at the active site of both the β_2 subunit and the $\alpha_2\beta_2$ complex.

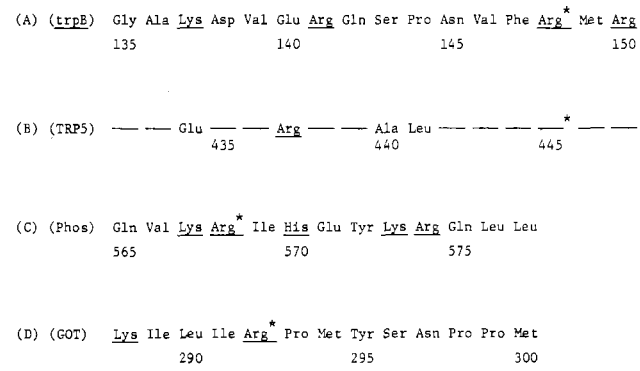

FIGURE 5: Amino acid sequences near active-site arginyl residues (Arg*) in pyridoxal phosphate enzymes. (A) *E. coli trpB* protein (Crawford et al., 1980). L-Serine binds to Arg-148. CB V contains residues 135–149. (B) Yeast TRP5 protein (Zalkin & Yanofsky, 1982). Residues identical with those in *E. coli* are indicated by dashes. Inactivation by phenylglyoxal has not been reported. (C) Glycogen phosphorylase (Phos) (Vandenbunder et al., 1981; Titani et al., 1977). Glucose 1-phosphate binds to Arg-568. (D) Mitochondrial aspartate aminotransferase (chicken) (GOT) (Sandmeier & Christen, 1982; Graf-Hausner, 1981; P. Christen, personal communication). The distal carboxyl of substrate binds Arg-292. Basic residues are underlined.

Functional arginyl residues which are modified by phenylglyoxal have been located in the primary sequences of only three pyridoxal phosphate enzymes; the sequences near these arginyl residues are shown in Figure 5. Each of these three phenylglyoxal-modifiable arginyl residues serves a different function. Arg-148 in the β_2 subunit binds the α-carboxyl group of L-serine (this report). Arg-292 in mitochondrial aspartate aminotransferase from chicken binds the distal carboxyl group of the substrate L-aspartate (Sandmeier & Christen, 1982). Arg-586 in glycogen phosphorylase binds the phosphate of the substrate glucose 1-phosphate (Vandenbunder et al., 1981). Although phenyglyoxal-modifiable residues in some pyridoxal phosphate enzymes bind the phosphate of the coenzyme (see the introduction), none of these arginyl residues has yet been located in the primary sequence.

The rather selective reaction of phenylglyoxal with Arg-148 of the β_2 subunit is probably due to the presence of this residue in an anion binding site. Patthy & Thész (1980) have proposed that the electric potential of anion binding sites may lower the pK_a value of an arginine in the site and thus make the arginine more reactive chemically. The presence of a cluster of several basic residues in an anion binding site may help to lower the pK_a value. The primary sequence of the β_2 subunit near Arg-148 contains three other basic residues: Lys-137, Arg-141, and Arg-150 (Figure 5). Arg-148 is conserved in the sequence of the β_2 subunit from *E. coli* and *S. typhimurium* (Crawford et al., 1980) and in the sequence of the enzyme from yeast at position 445; the yeast enzyme has both α and β activities in a single polypeptide chain (Zalkin & Yanofsky, 1982). The conservation of Arg-148 during evolution is consistent with its having an essential role.

Arg-141 and Arg-150 are also conserved in the sequences of the enzymes from *S. typhimurium* and from yeast. The yeast enzyme shows two complementary changes of acid and basic residues in this region: *E. coli* Lys-137 → yeast Glu-434 and *E. coli* Glu-140 → yeast Arg-437 (Figure 5). The fact that the enzymes from *E. coli*, *S. typhimurium*, and yeast all have the same numbers of basic (four) and acidic (two) residues in this region of the sequence may be significant, since Loeb & Saroff (1964) have postulated that clusters of protonated nitrogen centers (His, Lys, Arg) hydrogen bonded to carboxyl groups (Asp, Glu) may give rise to anion binding

sites. The proposal of these authors that several such clusters of three basic nitrogen centers plus two carboxyl groups exist in ribonuclease is supported by X-ray crystallographic studies of ribonuclease which show regions of high positive charge near the active site; Lys-7, Arg-10, and Arg-39 and His-12, Lys-41, and His-119 form two closely associated groups (Richards & Wyckoff, 1971).

The primary sequence of glycogen phosphorylase (Figure 5) near Arg-568 contains four other basic reesidues (Lys-567, His-570, Lys-573, and Arg-574) and one acidic residue (Glu-571). Crystallographic studies show that Lys-573 and Arg-568 are close to the phosphate of glucose 1-phosphate (Johnson et al., 1980). Arg-568 is hydrogen bonded to Asp-283 in the unreactive T conformation of the enzyme but may move away in the reactive R conformation (Vandenbunder et al., 1981). The primary sequence of aspartate aminotransferase near Arg-292 (Figure 5) contains only one other basic residue and no carboxyl groups and is thus quite different from the sequences of phosphorylase and of the β_2 subunit which contains four or more basic residues and one or more acidic residue near the functional arginine.

Our work contributes significantly to the knowledge of the active-site residues of the β_2 subunit since this is the first functional arginyl residue to be identified in the β_2 subunit and the first residue to be identified which is involved in substrate binding. Our recognition that Arg-148 and the cluster of basic residues in the primary sequence near Arg-148 have been conserved in evolution and probably serve in anion binding helps us to relate sequence to function.

Acknowledgments

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Supplementary Material Available

Figures showing competition between L-serine and phenylglyoxal under assay conditions (Figure 1), L-serine binding by the method of Hummel and Dreyer (Figure 2), and Hill plots of spectrophotometric titration data (Figure 3) (3 pages). Ordering information is given on any current masthead page.

Registry No. Tryptophan synthase, 9014-52-2; L-arginine, 74-79-3; L-serine, 56-45-1; L-tryptophan, 73-22-3; phenylglyoxal, 1074-12-0; aspartate aminotransferase, 9000-97-9; glycogen phosphorylase, 9035-74-9; D-tryptophan, 153-94-6; pyridoxal phosphate, 54-47-7.

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Effects of Metal Ion Substitution on Carboxypeptidase A Catalyzed Hydrolysis of *O-trans*-Cinnamoyl-L-β-phenyllactate†

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ABSTRACT: The effects of divalent metal ion substitution (Ni^{2+}, Co^{2+}, Cu^{2+}, and Hg^{2+}) for Zn^{2+} on the kinetics of carboxypeptidase A catalyzed hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate have been determined in H_2O at 30 °C. The Cu(II) enzyme is inactive at pH values in the range 5.78–9.45, and the Hg(II) enzyme is inactive at pH 7.50 and 9.80. The Zn(II), Ni(II), and Co(II) enzymes give similar plots of k_{cat}, K_m, and k_{cat}/K_m vs. pH. The values of K_m are nearly pH independent in the range 5–9 but increase markedly at pH >9. The bell-shaped plots of k_{cat}/K_m vs. pH give pK_{app}^{E} values of 9.0, which are invariant, and near 6.0, which differ only slightly. The k_{cat} vs. pH profiles all show a sigmoidal region in which pK_{app}^{ES} values are closely similar [Zn(II), 6.2; Ni(II), 6.2; Co(II), 5.7] and a rapidly rising arm at pH >9. Values of k_{cat} on the upper arm indicate rate enhancements of 10^7–10^8 over nonenzymatic OH^--catalyzed hydrolysis of the ester. At lower pH (6–9), k_{cat}(lim) differs but slightly in the order Ni(II) > Co(II) > Zn(II). Modification of Glu-270 to the methoxyamide results in loss of activity at all pH values, showing that the carboxylate group is necessary for the reaction even at pH >9. The plot of k_{cat} vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-mandelate is bell shaped. Values of k_{cat} are much less than those with *O-trans*-cinnamoyl-L-β-phenyllactate (230-fold at pH 8.20). These results indicate that (a) hydrolysis of an anhydride intermediate is rate determining at all pH values greater than 6 in hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate, including pH >9, but possibly not in hydrolysis of *O-trans*-cinnamoyl-L-mandelate, (b) the apparent pK_a^{ES} at pH 6 is not that of metal ion bound water but represents either a composite constant or a change in the rate-determining step, and (c) both formation and breakdown of the anhydride intermediate must be greatly facilitated by the enzyme.

Carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2)[1] is a Zn(II)-requiring enzyme that catalyzes the hydrolysis of peptides and *O*-acyl derivatives of α-hydroxy carboxylic acids (Hartsuck & Lipscomb, 1971). Binding of esters requires the presence of metal ion whereas binding of peptides does not (Auld & Holmquist, 1974). However, the presence of metal ion is required for catalytic activity with both types of substrate. The primary amino acid sequence has been determined (Bradshaw et al., 1969), and the three-dimensional crystal structure has been elucidated by X-ray crystallographic analysis at 2-Å resolution (Ludwig & Lipscomb, 1973; Lipscomb, 1970). The X-ray studies have shown the zinc ion to be chelated to the carbonyl oxygen of poor peptide substrates. The carboxyl group of glutamic acid-270 has also been implicated in the catalytic process, and mechanisms have been suggested involving nucleophilic attack and classical general-base catalysis [proton transfer in the transition state from a water molecule (Lipscomb, 1970)].

Much of what is known of the effects of the metal ion in carboxypeptidase A reactions has been obtained from metal ion substitution studies (Hartsuck & Lipscomb, 1971; Ludwig & Lipscomb, 1973). Replacement of Zn(II) in the active site by a large series of metal ions has been achieved (Vallee et al., 1958; Coleman & Vallee, 1960, 1961; Davies et al., 1968; Auld & Vallee, 1970a). The configuration of the Ni(II) enzyme has been shown to be octahedral (Rosenberg et al., 1975b), whereas the coordination geometries of cobalt and copper carboxypeptidase were determined to be five-coordinate and tetrahedral, respectively (Rosenberg et al., 1973, 1975a). From the X-ray work, it is known that carboxypeptidase-bound zinc exhibits a distorted tetrahedral geometry (Lipscomb, 1970). These metalloenzymes all apparently show at least some esterase activity toward *O*-hippuryl-DL-β-phenyllactate except the Cu(II) (Coleman & Vallee, 1961) and the Co(III) enzyme (Van Wart & Vallee, 1978). The studies of the effects of metal ion substitution have been carried out with various substrates and different reaction conditions so that direct comparisons are difficult.

A variety of k_{cat} vs. pH profiles have been observed for hydrolysis of ester substrates of carboxypeptidase A (Hall et al., 1969; Carson & Kaiser, 1966; Bunting et al., 1974). As

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[1] Abbreviations: CPA, bovine pancreatic carboxypeptidase A; CPL, *O-trans*-cinnamoyl-L-β-phenyllactate; CM, *O-trans*-cinnamoyl-L-mandelate; CoCPA, Co^{2+}-substituted carboxypeptidase A; NiCPA, Ni^{2+}-substituted carboxypeptidase A; ΔH_i, enthalpy of ionization; Tris, tris(hydroxymethyl)aminomethane.

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