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Photoinactivation and Photoaffinity Labeling of Tryptophan Synthase $\alpha_2\beta_2$ Complex by the Product Analogue 6-Azido-L-tryptophan

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ABSTRACT: The photoaffinity reagent 6-azido-L-tryptophan was synthesized by chemical methods. It binds reversibly in the dark to the $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli* and forms a quinonoid intermediate with enzyme-bound pyridoxal phosphate (λ_{max} = 476 nm). The absorbance of this chromophore has been used for spectrophotometric titrations to determine the binding of 6-azido-L-tryptophan (the half-saturation value $[S]_{0.5}$ = 6.3 μM). Photolysis of the quinonoid form of the $\alpha_2\beta_2$ complex results in time-dependent inactivation of the β_2 subunit but not of the α subunit. The extent of photoinactivation is directly proportional to the absorbance at 476 nm of the quinonoid intermediate prior to photolysis. The substrate L-serine is a competitive inhibitor of 6-azido-L-tryptophan binding and photoinactivation. The competitive inhibitors L-tryptophan, D-tryptophan, and oxindolyl-L-alanine also protect against photoinactivation. The results demonstrate that 6-azido-L-tryptophan is a quasi-substrate for the $\alpha_2\beta_2$ complex of tryptophan synthase and that photolysis of the enzyme–quasi-substrate quinonoid intermediate results in photoinactivation. The modified $\alpha_2\beta_2$ complex retains its ability to bind pyridoxal phosphate and to cleave indole-3-glycerol phosphate, a reaction catalyzed by the α subunit. 6-Azido-L-tryptophan (side-chain 1,2,3-$^{14}C_3$ labeled) was synthesized enzymatically from 6-azidoindole and uniformly labeled L-[^{14}C]serine by the $\alpha_2\beta_2$ complex of tryptophan synthase on a preparative scale and has been isolated. Incorporation of ^{14}C label from 6-azido-L-[^{14}C]tryptophan is stoichiometric with inactivation. Our finding that most of the incorporated ^{14}C label is bound in an unstable linkage suggests that an active site carboxyl residue is the major site of photoaffinity labeling by 6-azido-L-tryptophan.

Tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.1.2.20) from *Escherichia coli* catalyzes the synthesis of L-tryptophan from L-serine and indole-3-glycerol phosphate (reaction 1) or from L-serine and indole (reaction 2), as well as the cleavage of indole-3-glycerol phosphate (reaction 3). Reactions 2 and 3 are catalyzed at the active sites of the β_2 subunit and of the α subunit, respectively. [For reviews of tryptophan synthase, see Yanofsky & Crawford (1972) and Miles (1979).]

indole-3-glycerol phosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O (1)

indole + L-serine → L-tryptophan + H_2O (2)

indole-3-glycerol phosphate ⇌ indole + D-glyceraldehyde 3-phosphate (3)

Although the $\alpha_2\beta_2$ complex of tryptophan synthase does not catalyze degradation of L-tryptophan, it does bind L-tryptophan and forms a pyridoxal phosphate intermediate with L-tryptophan, which has an absorbance maximum at 476 nm (Miles, 1980; Tschopp & Kirschner, 1980; Lane & Kirschner, 1981; Tanizawa & Miles, 1983; Phillips et al., 1984). This intermediate probably has a quinonoid structure and results from removal of the α-proton of L-tryptophan from the Schiff-base intermediate. Similar quinonoid intermediates have been observed with oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan, which are potent competitive inhibitors of the enzyme (Phillips et al., 1984). Tryptophan synthase catalyzes α-hydrogen exchange of L-tryptophan in ^2H_2O (Tsai et al., 1978; Miles, 1980) and of oxindolyl-L-alanine in ^3H_2O (Phillips et al., 1984).

Several active site residues have been previously identified in the β_2 subunit of tryptophan synthase. These include

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Lys-87, which forms a Schiff base with pyridoxal phosphate, His-82 or His-86, which removes the α-hydrogen of L-serine, Arg-148, which binds the carboxyl of L-serine, and Cys-230 (Higgins et al., 1980; Crawford et al., 1980; Miles & Higgins, 1980; Tanizawa & Miles, 1983).

In order to further characterize substrate binding groups or catalytic groups in the tryptophan binding site of the tryptophan synthase $\alpha_2\beta_2$ complex, we have synthesized 6-azido-L-tryptophan both chemically and enzymatically. The 6-position was chosen for introducing the azido group since indoles modified at the 6-position are known to be substrates for the *Neurospora crassa* tryptophan synthase (Hall et al., 1962; Saito & Rilling, 1982). 6-Azido-L-tryptophan is an aryl azide, which are the most commonly used type of photoactivatable reagent (Bayley & Knowles, 1977; Chowdry & Westheimer, 1979; Bayley, 1983). 6-Azido-L-tryptophan has not been previously tested as a photoaffinity label. Our results establish that 6-azido-L-tryptophan binds reversibly in the dark to the $\alpha_2\beta_2$ complex of tryptophan synthase and is a remarkably efficient photoinactivator and photoaffinity label of the β_2 subunit.

Experimental Procedures

Materials. Indole-3-propanol phosphate was a generous gift of Dr. Kasper Kirschner (Kirschner et al., 1975). Oxindolyl-L-alanine was prepared as described by Phillips et al. (1984). 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 and Yanofsky, 1965). Uniformly labeled L-[^{14}C]serine (152 mCi/mol) was obtained from New England Nuclear. 6-Azidoindole was synthesized by the method of Melhado & Leonard (1983). The chemical synthesis of 6-azido-L-tryptophan is described under Results. 6-Azido-L-tryptophan (side-chain 1,2,3-$^{14}C_3$ labeled) was synthesized by incubating 2 μmol of L-serine, containing 10 μCi of uniformly labeled L-[^{14}C]serine, 2 μmol of 6-azidoindole, and 20 nmol of tryptophan synthase $\alpha_2\beta_2$ complex in a final volume of 1.0 mL of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.0, for 30 min at 25 °C. After addition of 60 μL of glacial acetic acid to precipitate protein, the solution was centrifuged for 1 min in a Beckman microfuge B, and the supernatant solution was applied to a column of Sephadex G-25 superfine (0.8 cm × 69 cm) and eluted with water at 2 mL/h, collecting 0.86-mL fractions (Saito & Rilling, 1981). 6-Azido-L-tryptophan (side-chain 1,2,3-$^{14}C_3$ labeled) eluted after 86 mL, about 3 times the void volume (27 mL). The product was obtained in about 50% yield and gave a single spot (R_f 0.4) upon thin-layer chromatography (TLC) on reverse-phase plates (Analtech RPS-F 250 μm) in 25% methanol–75% water. Solutions of 6-azido-L-tryptophan were handled in dim light and stored at −20 °C in containers covered with aluminum foil.

Enzymes and Enzyme Assays. The tryptophan synthase $\alpha_2\beta_2$ complex from *E. coli* strain *W3110 trpRcysBΔtrpLD102trpB$^+$trpA$^+$/F'colVBcysB$^+$-ΔtrpLD102trpB$^+$trpA$^+$* was prepared 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 protein method of Lowry et al. (1951) with the unmodified protein serving as a standard. Reaction 2 ("the β_2 reaction") was measured spectrophotometrically (Higgins et al., 1979). Reaction 1 ("the $\alpha\beta$ reaction") and reaction 3 ("the α reaction") were measured by spectrophotometric assays coupled with glyceraldehyde-3-phosphate dehydrogenase as described by Creighton & Yanofsky (1966). One unit of activity is defined as the conversion of 0.1 μmol of substrate to product per 20 min at 37 °C.

Spectrophotometric Titration of the $\alpha_2\beta_2$ Complex with Amino Acids. The absorbance at 476 nm of solutions of holo-$\alpha_2\beta_2$ complex (0.01 mM in $\alpha\beta$ monomer in 0.1 M Tris-HCl, pH 8.0) was determined with a Cary 118 spectrophotometer at 23 °C after each addition of 6-azido-L-tryptophan; 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 were then plotted as log $[R/(1 - R)]$ vs. 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). The half-saturation values ($[S]_{0.5}$) were determined from the plots where log $[R/(1 - R)] = 0$.

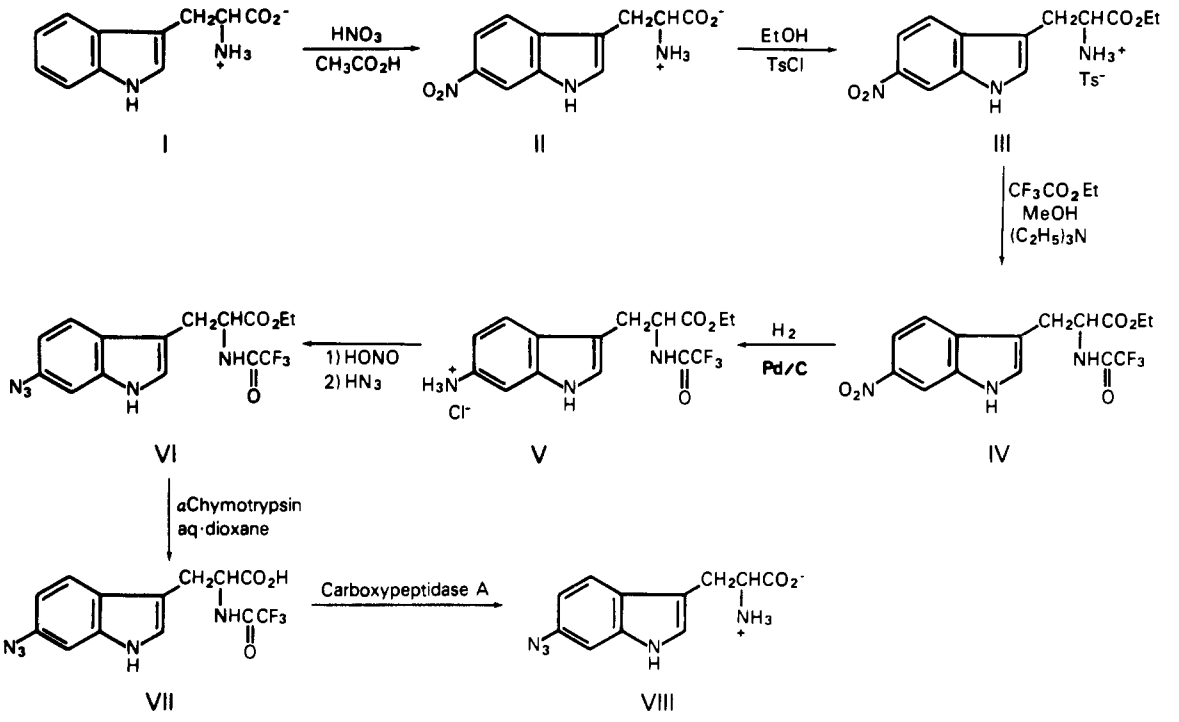
Photolysis Conditions. Photolysis was carried out at 22 °C in a quartz cuvette (0.2-cm light path) irradiated by a Mineralight lamp Model UVGL-25 at a distance of 5 cm. The lamp was turned on 5 min before the cuvette was put in the lamp beam. In time-course experiments, the lamp was left on throughout, and the cuvette was removed at intervals for measuring spectra in a Cary 118 spectrophotometer or for taking aliquots for assay. Photoinactivation reaction mixtures contained tryptophan synthase $\alpha_2\beta_2$ complex (0.01–0.05 mM) in 0.1 M Tris-HCl, pH 8.0, containing 10 mM β-mercaptoethanol, 0.1 mM indole-3-propanol phosphate, and 0.1 mM 6-azido-L-tryptophan, unless otherwise indicated.

Measurement of Bound Ligands. Protein-bound ^{14}C label was measured by a slight variation of the centrifuged-column procedure of Penefsky (1979). Columns of Sephadex G-25 fine (Pharmacia) (final bed volume 0.8 mL after centrifugation as below) were prepared in disposable, 1-mL plastic tuberculin syringes plugged with glass wool and suspended in centrifuge tubes. The columns were preequilibrated with the desired buffer by adding 0.2 mL of buffer and were centrifuged for 2.0 min at 300 rpm in a fixed-angle clinical centrifuge at room temperature. This procedure was repeated 2 more times. Samples (0.2 mL) were applied to columns and centrifuged as above in empty tubes. The excluded volume (about 0.2 mL) was diluted with 0.1 or 0.2 mL of buffer and used for determination of radioactivity, enzyme activity, and protein concentration. In some experiments 4-mL centrifuge columns were prepared in 5-mL syringes and treated with 1.0-mL volumes of buffer or of samples as described above. Radioactivity was measured in a Beckman LS-8100 instrument with Aquasol as scintillation fluid.

Results

Synthesis of 6-Azido-L-tryptophan. The synthesis of 6-azido-L-tryptophan was carried out as shown in Scheme I. L-Tryptophan (I) was nitrated as described by De Fazi et al. (1959) to give 6-nitro-L-tryptophan (II), which was converted to the ethyl ester *p*-toluenesulfonate (III) by refluxing in absolute ethanol containing a slight excess of *p*-toluenesulfonyl chloride (TsCl) (Arai & Muramatsu, 1983). The ethyl ester was trifluoroacetylated by reaction with ethyl trifluoroacetate in methanol, as described by Curphey (1979) for L-tryptophan. The nitro group was reduced with 40 psi H_2 over Pd/C, and the amino product (V) was isolated as the hydrochloride salt. For conversion of the 6-amino to the 6-azido derivative, 0.296

Scheme I



g of V (0.78 mmol) was suspended in 32 mL of 0.1 M HCl and cooled on ice, and 59 mg of $NaNO_2$ in 0.5 mL of H_2O was added with vigorous stirring. After 5 min, 76 mg of NaN_3 in 1 mL of H_2O was added, and after N_2 evolution had ceased, the reaction mixture was extracted twice with 30 mL of ethyl acetate. The extract was dried over Na_2SO_4, concentrated in vacuo, and applied to a silica gel preparative TLC plate (1500 μm thickness). After elution with ethyl acetate/hexanes (1:2), the major UV-absorbing band was scraped off and eluted from the silica with ethyl acetate, and the product, VI, was crystallized from ethyl acetate/petroleum ether: yield, 0.156 g (54%) of cream needles; mp 123–124 °C; UV λ_{max} (95% EtOH) 217 nm (ϵ 2.2 × 10^4), 248 (ϵ 2.4 × 10^4), 292 (ϵ 1.1 × 10^4); mass spectrum (CI, NH_3), *m/e* (relative intensity) 387 (35) (M + 18), 370 (85) (M + 1), 342 (100) (M + 1 − N_2); IR ($CHCl_3$) 2110 cm^{-1}. Anal. Calcd for $C_{15}H_{14}F_3N_5O_3$: C, 48.78; H, 3.82; N, 18.96. Found: C, 48.87; H, 3.61; N, 18.91.

The protecting groups were removed enzymatically, as we have described previously (Phillips & Cohen, 1983) for other tryptophan derivatives. In a typical experiment, 70 mg of VI dissolved in 1.5 mL of dioxane was added to a solution of 15 mg of α-chymotrypsin in a mixture of 6 mL of H_2O, 0.28 mL of 1 M $NaHCO_3$, and 1.5 mL of dioxane. The flask was covered with foil and stirred at room temperature. Much of the substrate precipitated, so the reaction was allowed to proceed until no precipitate was apparent and TLC analysis indicated complete hydrolysis of the ester (1 week). The reaction mixture was then frozen in dry ice/acetone and lyophilized. The dried residue was extracted with 30 mL of methanol, and the extract was filtered through celite to remove precipitated protein. After removal of the methanol in vacuo, the residue was dissolved in 25 mL of water and extracted with 15 mL of ethyl acetate. The aqueous layer was then adjusted to pH 2 with 1 N HCl and extracted with 5 × 15-mL portions of ethyl acetate. The combined extracts were dried over Na_2SO_4, and the ethyl acetate was removed in vacuo, giving 60.8 mg of VII (93%) as a tan solid. This product was suspended in 4 mL of H_2O, and the pH was adjusted to about 8 with 29.5 μL of triethylamine. A total of 2.2 mg of carboxypeptidase A was added, and the reaction was allowed to proceed for 18 h. The reaction mixture was then frozen and lyophilized, and the residue was extracted with 40 mL of methanol. The methanol extracts were filtered through celite, and the solvent was removed in vacuo. Trituration of the residue with 5 mL of $CHCl_3$/methanol (1:1) gave 26.5 mg (60.7%) of 6-azido-L-tryptophan (VII) as a powdery white solid: UV λ_{max} (H_2O) 215 nm (ϵ 1.78 × 10^4), 246 nm (ϵ 2.04 × 10^4), 288 nm (ϵ 0.88 × 10^4); mass spectrum (CI, NH_3), *m/e* (relative intensity) 246 (15) (M + 1); IR (KBr) 2110 cm^{-1}; mp 185–190 °C dec. 6-Azido-L-tryptophan (side-chain 1,2,3-$^{14}C_3$ labeled) was synthesized enzymatically as described under Experimental Procedures. This product was identical with the chemically synthesized 6-azido-L-tryptophan in its UV spectral properties (A_{246nm}/A_{288nm} = 2.3), had an identical R_f upon TLC (see Experimental Procedures), and gave identical results in the experiments with tryptophan synthase described below.

Photolysis of 6-Azido-L-tryptophan. Figure 1 shows the effect of irradiation for several time periods on the absorption spectrum of 6-azido-L-tryptophan. The bands at 247 and at 290 nm are rapidly abolished during irradiation. The rate of photolysis follows first-order kinetics (see inset of Figure 1) with a half-time of 12 s under these conditions. A peak at 300 nm is seen after irradiation. The products of photolysis have not been identified. Since addition of 0.01 M β-mercaptoethanol to solutions of 6-azido-L-tryptophan caused no change in the absorption spectrum (data not shown), we conclude that 6-azido-L-tryptophan is not easily reduced under these conditions. Some aryl azides are reduced by thiols under comparable conditions (Staros et al., 1978).

Reversible Binding of 6-Azido-L-tryptophan by Tryptophan Synthase. Figure 2A shows the effects of adding 0.1 mM 6-azido-L-tryptophan to the $\alpha_2\beta_2$ complex of tryptophan synthase in the absence (curve 1) and presence (curve 2) of 0.1 mM indole-3-propanol phosphate, an analogue of indole-3-glycerol phosphate that binds to the active site of the α subunit (Kirschner et al., 1975). The peak observed at 476 nm in the presence of 6-azido-L-tryptophan and indole-3-propanol

Table I: Effect of Substrates and Other Additives upon Photoinactivation of the $\alpha_2\beta_2$ Complex by 6-Azido-L-tryptophan[a]

expt no.	primary addition (A)	other addition (B)	activity remaining (%)	protection (%)	K_D of analogue (μM) −IPP	K_D of analogue (μM) +IPP
1	none		96			
2	IPP[b]		97			
3	6-N_3-L-Trp		68		14 (text)	6.3 (Figure 2B)
4	6-N_3-L-Trp + IPP		30–39			
5	6-N_3-L-Trp + IPP (prephotolyzed)[c]		100			
6	6-N_3-L-Trp + IPP	1 mM sodium azide	23			
7	6-N_3-L-Trp + IPP	0.1 mM L-tryptophan	51	20	90[d]	53[e]
8	6-N_3-L-Trp + IPP	1.0 mM L-tryptophan	76	64	80[f]	36[h]
9	6-N_3-L-Trp + IPP	0.1 mM D-tryptophan	86	77	30[g]	6[h]
10	6-N_3-L-Trp + IPP	1.0 mM D-tryptophan	97	95	15[e]	
11	6-N_3-L-Trp + IPP	0.1 mM oxindolyl-L-alanine	100	100	5[f]	
12	6-N_3-L-Trp + IPP	1.0 mM oxindolyl-L-alanine	100	100		
13	6-N_3-L-Trp + IPP	1.0 mM L-serine	87	79	9[h]	
14	6-N_3-L-Trp + IPP	10 mM L-serine	97	97	10[i]	1.1[i]
15	6-N_3-L-Trp + IPP	1.0 mM DL-alanine	33	0		
16	6-N_3-L-Trp + IPP	10 mM DL-alanine	41	8		

[a] A total of 0.01 mM $\alpha_2\beta_2$ complex in 0.1 M Tris-HCl, pH 8.0, containing 0.01 M β-mercaptoethanol, in the presence or absence of 0.1 mM indole-3-propanol phosphate and 0.1 mM 6-azido-L-tryptophan indicated as primary addition (A) and other addition (B), was assayed for activity in reaction 2 before and after irradiation for 5 min at 5 cm in 0.2-cm path-length cell. The percent of the initial activity of each solution before irradiation is given. The initial activity of samples in the presence of some substrate analogues was between 50% and 100% of the activity of the control with no addition. An initial lag of less than 1 min was observed in assays in the presence of D-tryptophan and oxindolyl-L-alanine, which are slowly released inhibitors. Percent protection = 100 × (percent activity remaining − percent activity of control remaining/100% activity of control remaining), where the control is the enzyme in experiment 4; the activity of the control remaining varied from 30% to 39% in different experiments. [b] Abbreviations used are as follows: 6-N_3-L-Trp, 6-azido-L-tryptophan; IPP, indole-3-propanol phosphate. [c] 6-Azido-L-tryptophan was prephotolyzed for 5 min in the absence of enzyme, which was then added. [d] Tschopp & Kirschner, 1980. [e] Lane & Kirschner, 1981. [f] Phillips et al., 1984. [g] Tanizawa & Miles, 1984. [h] Miles, 1980. [i] Lane & Kirschner, 1983.

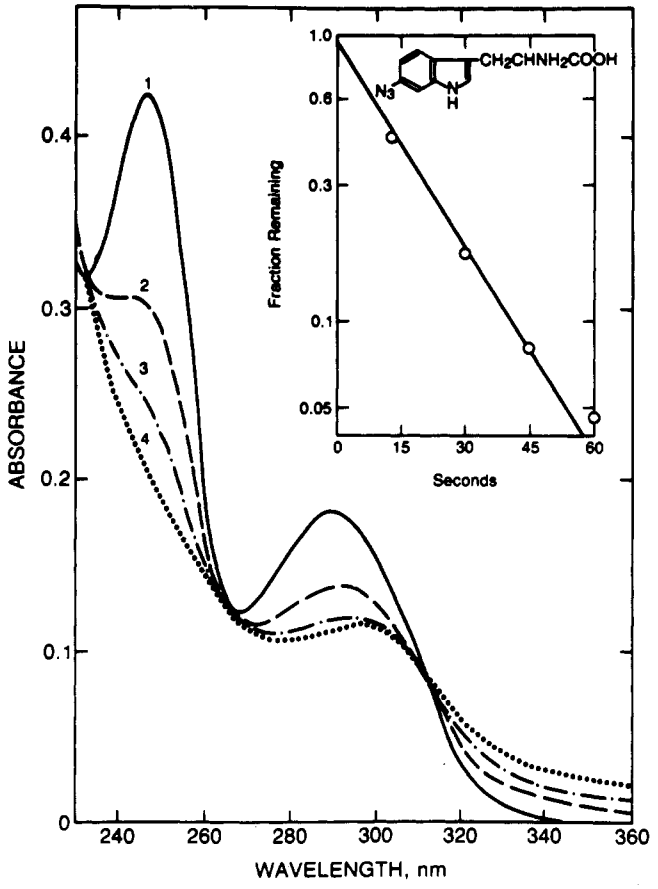


FIGURE 1: Effect of irradiation on the absorption spectrum of solutions of 6-azido-L-tryptophan. 6-Azido-L-tryptophan (0.10 mM in 0.1 M Tris-HCl, pH 8.0) was irradiated as described under Experimental Procedures in a 0.2-cm path-length cuvette. At various times the cuvette was removed, and the spectrum was recorded: curve 1 (t = 0); curve 2 (t = 15 s); curve 3 (t = 30 s); curve 4 (t = 2, 3, 4, or 5 min). The inset shows the fraction remaining as a function of the time of irradiation. The fraction remaining is determined from the following relationship: (A_{247nm} at $t = X - A_{247nm}$ at t = 5 min)/(A_{247nm} at t = 0 − A_{247nm} at t = 5 min).

phosphate is essentially identical with one that has been observed upon addition of L-tryptophan and indole-3-propanol phosphate and that has been ascribed to a quinonoid intermediate (Miles, 1980; Lane & Kirchner, 1981). Thus the intermediate formed with 6-azido-L-tryptophan probably has the quinonoid structure shown in Figure 2A. Indole-3-propanol phosphate appears to increase the steady-state level of this intermediate with both L-tryptophan (Miles et al., 1980; Lane & Kirschner, 1981) and 6-azido-L-tryptophan (Figure 2A). The absorbance at 476 nm of the chromophore formed with L-tryptophan and with 6-azido-L-tryptophan has been used for spectrophotometric titrations in the presence and absence of indole-3-propanol phosphate to determine the binding of L-tryptophan (Miles, 1980; Tschopp & Kirschner, 1980; Phillips et al., 1984; Lane & Kirschner, 1981) and of 6-azido-L-tryptophan (see below and Table I). In separate kinetic experiments, 6-azido-L-tryptophan was found to be a competitive inhibitor of L-serine in the β_2 reaction (reaction 2) with a K_I of about 200 μM (data not shown; see Experimental Procedures for the assay method). Although our finding that the K_I value for 6-azido-L-tryptophan (200 μM) is much higher than the K_D value (14 μM) (see Table I) in the absence of indole-3-propanol phosphate is surprising, similar discrepancies between K_I and K_D values have been reported for this enzyme with L-tryptophan (Phillips et al., 1984) and L-serine (Lane & Kirschner, 1983). 6-Azido-L-tryptophan binds more tightly than L-tryptophan in either the presence or absence of indole-3-propanol phosphate (Table I).

Competition between L-Serine and 6-Azido-L-tryptophan. L-Serine and 6-azido-L-tryptophan compete for the same binding site, since addition of L-serine to $\alpha_2\beta_2$ complex previously treated with 6-azido-L-tryptophan in the presence of indole-3-propanol phosphate (Figure 2A, curve 2) displaces the peak at 476 nm (Figure 2A, curve 3) and gives the same spectrum obtained with L-serine alone in the presence of indole-3-propanol phosphate. A reciprocal plot of the data from spectrophotometric titrations of the $\alpha_2\beta_2$ complex with 6-azido-L-tryptophan in the presence and absence of 5 or 10 μM

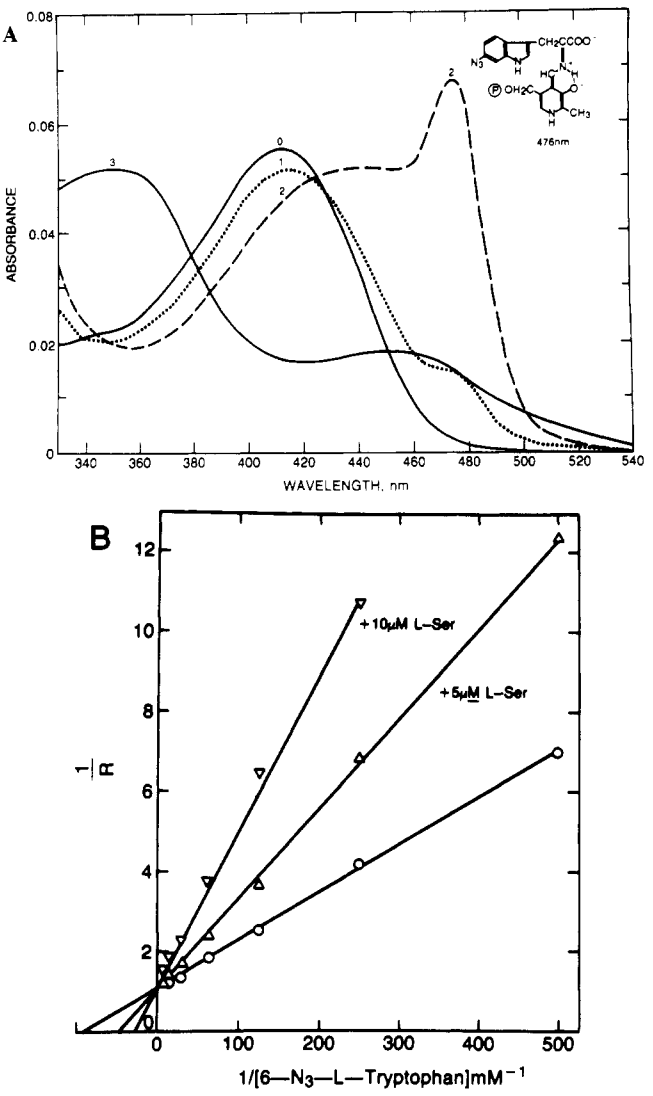


FIGURE 2: Effect of 6-azido-L-tryptophan and L-serine upon the absorption spectra (A) and upon the absorbance at 476 nm (B) of the $\alpha_2\beta_2$ complex of tryptophan synthase. (A) Absorption spectra of the $\alpha_2\beta_2$ complex of tryptophan synthase (0.01 mM in $\alpha\beta$ protomer in 0.1 M Tris-HCl buffer, pH 7.8) before (curve 0) and after the addition of 0.1 mM 6-azido-L-tryptophan (curve 1), 0.1 mM 6-azido-L-tryptophan and 0.1 mM indole-3-propanol phosphate, and 10 mM L-serine or 0.1 mM indole-3-propanol phosphate and 10 mM L-serine (curve 3). (B) The absorbance at 476 nm of the $\alpha_2\beta_2$ complex was determined as in (A) in the presence of 0.1 mM indole-3-propanol phosphate and of different concentrations of 6-azido-L-tryptophan (0.002–0.246 mM) and in the presence and absence of 5 or 10 μM L-serine. The value $R = \Delta\epsilon/\Delta\epsilon_{max}$ was calculated as described under Experimental Procedures, and $1/R$ is plotted vs. $1/$[6-azido-L-tryptophan] (mM^{-1}) to show the competition for binding by L-serine. The half-saturation value for 6-azido-L-tryptophan binding ($[S]_{0.5}$ = 6.3 μM) was determined from a further plot of log $R/(1-R)$ vs. log C_{free} as described under Experimental Procedures (data not shown).

L-serine (Figure 2B) shows that L-serine is indeed a potent competitive inhibitor of 6-azido-L-tryptophan binding. The half-saturation value, $[S]_{0.5}$, for binding of 6-azido-L-tryptophan to the $\alpha_2\beta_2$ complex was determined from Hill plots of the spectral titration data [see Experimental Procedures and Phillips et al. (1984) for method]. The $[S]_{0.5}$ values for 6-azido-L-tryptophan in the presence of indole-3-propanol phosphate were 6.3 μM in the absence of L-serine and 19 μM in the presence of 5 μM L-serine. The competitive inhibitor constant for L-serine (K_I = 2.5 μM) derived from these data is close to the dissociation constant (K_D = 1.1 μM) determined by spectrophotometric titration in the presence of indole-3-

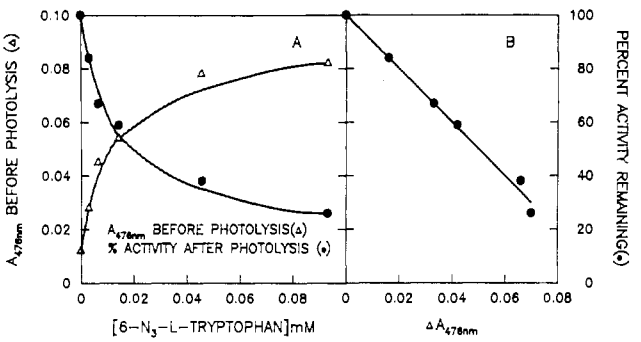


FIGURE 3: Relationship between concentration of 6-azido-L-tryptophan, absorbance of the $\alpha_2\beta_2$ complex at 476 nm, and the extent of photoinactivation. Tryptophan synthase $\alpha_2\beta_2$ complex (0.01 mM) was treated with the indicated concentrations of 6-azido-L-tryptophan in the presence of 0.1 mM indole-3-propanol phosphate as described under Experimental Procedures. The absorbance of each solution was recorded before irradiation. The activity of each solution in reaction 2 was determined before and after irradiation for 5 min. The percent activity remaining (●) and the change in absorbance at 476 nm ΔA_{476nm} (Δ) are plotted vs. the concentration of 6-azido-L-tryptophan in (A). The percent activity remaining (●) is plotted vs. the ΔA_{476nm} in (B).

propanol phosphate (Lane & Kirschner, 1983; see Table I).

Photoinactivation of Tryptophan Synthase by 6-Azido-L-tryptophan. When the $\alpha_2\beta_2$ complex of tryptophan synthase was photolyzed under the conditions given in Table I, maximal photoinactivation was observed in the presence of both 0.1 mM 6-azido-L-tryptophan and 0.1 mM indole-3-propanol phosphate (experiment 4). Less photoinactivation was observed in the presence of 0.1 mM 6-azido-L-tryptophan alone (experiment 3). Little or no inhibition was observed with no addition (experiment 1), with indole-3-propanol phosphate alone (experiment 2), or with prephotolyzed 6-azido-L-tryptophan (experiment 5). Sodium azide, which traps singlet oxygen, showed no protection (experiment 6).

Protection from Photoinactivation by Substrates and Quasi-Substrates. Addition of L-serine, L-tryptophan, D-tryptophan, or oxindolyl-L-alanine to the $\alpha_2\beta_2$ complex results in the formation of stable enzyme–substrate or enzyme–quasi-substrate intermediates, which can be observed spectrophotometrically (Miles, 1980; Lane & Kirschner, 1981, 1983; Tanizawa & Miles, 1983; Phillips et al., 1984). These four amino acids significantly protect the $\alpha_2\beta_2$ complex from photoinactivation by 6-azido-L-tryptphan (experiments 7–14). The concentration at which each of the aromatic amino acids provides protection is in the range to be expected from the binding constant (Table I, right column). L-Serine was used at a much higher concentration than its binding constant since this amino acid is converted by the $\alpha_2\beta_2$ complex to *S*-(hydroxyethyl)-L-cysteine in the presence of the β-mercaptoethanol (Goldberg & Baldwin, 1967; Miles et al., 1968). L-Serine is therefore present at a lower effective concentration in the photoinactivation reaction mixtures, which also contain β-mercaptoethanol and $\alpha_2\beta_2$ complex. The nonreactive L-alanine shows no protection (experiments 15 and 16).

Effects of 6-Azido-L-tryptophan Concentration upon Inactivation. Both the extent of photoinactivation and the extent of formation of the quinonoid intermediate (ΔA_{476nm}) show the same dependence upon 6-azido-L-tryptophan concentration (Figure 3A). Both 6-azido-L-tryptophan binding and photoinactivation are saturable and have the same half-maximally effective ligand concentration (0.02 mM). This value is not equal to a binding constant since a significant amount of the 6-azido-L-tryptophan is bound to the enzyme (see above). The extent of photoinactivation is directly proportional to the ab-

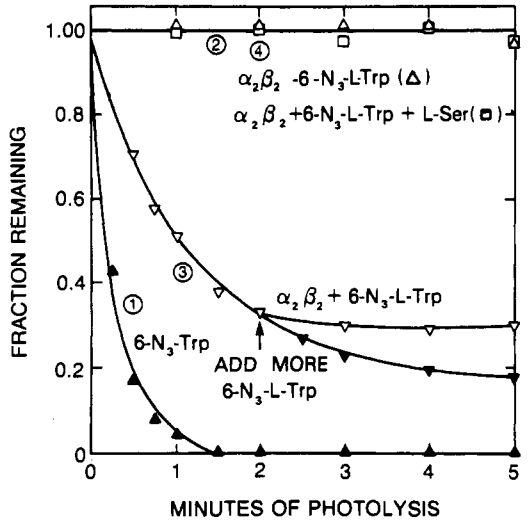


FIGURE 4: Time course of photolysis of 6-azido-L-tryptophan and photoinactivation of tryptophan synthase by 6-azido-L-tryptophan. 6-Azido-L-tryptophan was irradiated as described in Figure 1. The percent of the maximum observed change in absorbance at 247 nm is plotted vs. time of irradiation (curve 1, ▲). Tryptophan synthase $\alpha_2\beta_2$ complex was irradiated under conditions described in Table I and assayed for activity in reaction 2 after each time interval of irradiation. Curve 2 (△), no addition; curve 3 (▽), 0.1 mM 6-azido-L-tryptophan and 0.1 mM indole-3-propanol phosphate; curve 4 (□), 0.1 mM 6-azido-L-tryptophan, 0.1 mM indole-3-propanol phosphate, and 10 mM L-serine. The effects of a second addition of 0.1 mM 6-azido-L-tryptophan after 2 min (see the arrow on curve 3) on activity are shown by solid symbols (▼).

sorbance at 476 nm (Figure 3B). These results indicate that photoinactivation proceeds principally or exclusively through the quinonoid complex formed between enzyme-bound pyridoxal phosphate and 6-azido-L-tryptophan.

Time Course of Photoinactivation. Figure 4 shows the time course of photolysis of 6-azido-L-tryptophan (curve 1) and of photoinactivation of tryptophan synthase in the absence of 6-azido-L-tryptophan (curve 2), in the presence of 6-azido-L-tryptophan (curve 3), and in the presence of 6-azido-L-tryptophan and L-serine (curve 4). L-Serine provides complete protection against inactivation. Our finding that complete inactivation did not occur in the presence of nearly saturating 6-azido-L-tryptophan (0.1 mM) suggests that some of the enzyme-bound 6-azido-L-tryptophan was photolyzed without modifying and inactivating the enzyme. The absorbance at 476 nm of the enzyme used in curve 3 decreased in parallel to the decrease in activity (data not shown); both absorbance and activity reached minima after 2 min, a time at which free 6-azido-L-tryptophan had been completely photolyzed. A second addition of 0.1 mM 6-azido-L-tryptophan after 2 min of photolysis (arrow on curve 3) produced an increase in absorbance at 476 nm (data not shown) and increased inactivation after additional photolysis.

Determination of the Extent of Incorporation of ^{14}C Label. The extent of modification was determined from the amount of ^{14}C label bound to the $\alpha_2\beta_2$ complex after photolysis in the presence of different concentrations of 6-azido-L-[^{14}C]tryptophan (Figure 5). No ^{14}C label was bound in the absence of photolysis or in the presence of prephotolyzed reagent. A plot of the extent of incorporation vs. the percent activity remaining (Figure 5) is linear. Extrapolation of the line shows that the incorporation of 0.9 mol of ^{14}C label/mol of $\alpha\beta$ results in total inactivation. This stoichiometry and the finding that the data fall on a straight line indicate that modification of a single essential residue results in inactivation (Tsou, 1962).

Stability of the Incorporated ^{14}C Label. Most of the ^{14}C label bound to the $\alpha_2\beta_2$ complex after photolysis in the presence of 6-azido-L-[^{14}C]tryptophan is slowly released at pH 7.0 in the absence of urea ($t_{1/2} \simeq 24$ h) but is rapidly released at pH 7.0 or pH 8.8 in the presence of 8 M urea; release of label is even more rapid at pH 5.3 in the presence of 8 M urea (Figure 6A and Table II). Most of the activity that is lost upon photoinactivation is regained in parallel with the dissociation of the ^{14}C label in 8 M urea at pH 7.0 or pH 8.8 but not at pH 5.3 (Figure 6B). Reactivation also occurs upon prolonged incubation at pH 7.0 (Table II) (see Discussion). About 10% of the incorporated ^{14}C label is retained after prolonged incubation at pH 7.0 (Table II), after treatment with 8 M urea at pH 5.3 for 60 min, or after precipitation of the protein with acetone/HCl or with 1 N HCl (Table II). After heat precipitation of the α subunit by the procedure of Högberg-Raibaud and Goldberg (1977) (Table II), about half of the incorporated ^{14}C label is retained in the β_2 subunit; no label is found in the α subunit. The stability at pH 7.0 of the

Table II: Stability of the ^{14}C Label Incorporated into the $\alpha_2\beta_2$ Complex of Tryptophan Synthase after Photolysis in the Presence of 6-Azido-L-[^{14}C]tryptophan[a]

treatment after photolysis	time of incubation (h)	mol of ^{14}C/mol of subunit	activity (% of control)[g]
pH 7.0[b]	0	0.56/$\alpha\beta$	31
	24	0.27/$\alpha\beta$	53
	48	0.14/$\alpha\beta$	71
	96	0.10/$\alpha\beta$	77
heat treatment to precipitate	0	0.29/β	57
α subunit[c]	24	0.10/β	*h*
	0	0.01/α	*h*
8 M urea, pH 5.3[d]	1	0.07/$\alpha\beta$	42
1 N HCl[e]	0	0.062	*h*
acetone–HCl[f]	0	0.065	*h*

[a] Tryptophan synthase $\alpha_2\beta_2$ complex was photolyzed in the presence (modified) or absence (control) of 6-azido-L-[^{14}C]tryptophan as described in Figure 6. [b] The photolyzed solutions (1.0 mL) were applied to 4-mL centrifuge columns in 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM β-mercaptoethanol and 2 mM ethylenediaminetetraacetic acid (EDTA) (buffer A) at t = 0; 0.2-mL aliquots of the filtrates were applied to 0.8-mL centrifuge columns in the same buffer without β-mercaptoethanol (buffer B) at t = 0, 24, 48, or 96 h and assayed for absorbance at 278 nm, activity, and radioactivity as described under Experimental Procedures. [c] The photolyzed enzyme solutions (1.0 mL) were applied to 4-mL centrifuge columns in 0.1 M potassium phosphate buffer, pH 7.8, containing 10 mM β-mercaptoethanol, 2 mM EDTA, and 0.1 mM pyridoxal phosphate. The filtrates were heated for 10 min at 63 °C and centrifuged for 15 min at 10000 rpm. The supernatant solutions were applied to 4-mL centrifuge columns in buffer A (see footnote *b*); 0.2-mL aliquots of the filtrates were applied to 0.8-mL centrifuge columns in buffer B and assayed (see footnote *b*). The precipitates from the heat step were washed 2 times by resuspension in 1 mL of buffer B and centrifuged for 15 min at 10000 rpm. The washed precipitates were dissolved in 0.4 mL of 8 M urea in buffer B and assayed for absorbance at 278 nm and radioactivity. [d] Treatment and data from Figure 6. [e] Filtrates (0.2 mL) in buffer B prepared as described in footnote *b* were treated with 0.2 mL of 2 N HCl for 15 min at 4 °C and centrifuged for 1 min in a Beckman microfuge B. Each precipitate was washed 3 times by resuspension in 1 mL 1 N HCl for 15 min at 4 °C followed by centrifugation as above. Each washed precipitate was dissolved in 0.4 mL of 8 M urea in buffer B and assayed for absorbance at 278 nm and radioactivity. [f] Filtrates (0.2 mL) in buffer B prepared as described in footnote *b* were treated with 1.8 mL of a solution containing 39 volumes of acetone and 1 volume of 1 N HCl for 15 min at 4 °C and were then centrifuged for 1 min in a Beckman microfuge B. Each precipitate was washed 3 times by resuspension in 1 mL of acetone–HCl followed by centrifugation as above. Each washed precipitate was dissolved in 0.4 mL of 8 M urea in buffer B and assayed for absorbance at 278 nm and radioactivity. [g] The β_2 activity of modified and control enzymes in reaction 2 was measured in the presence of excess α subunit. The specific activities of the control solutions of $\alpha_2\beta_2$ complex and of β_2 subunit were 1000 and 1900 units/mg, respectively. [h] Not determined.

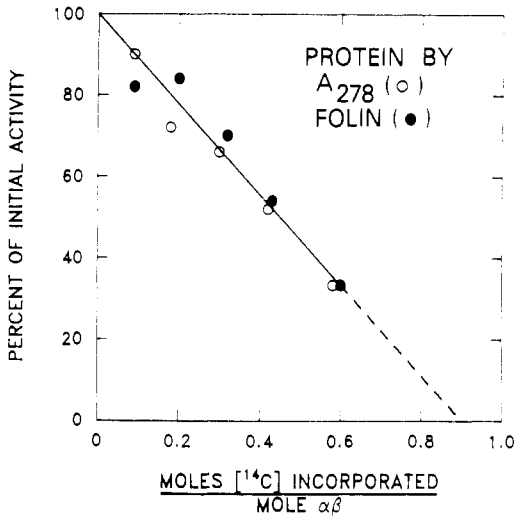


FIGURE 5: Effect of the extent of modification upon the residual activity. Tryptophan synthase $\alpha_2\beta_2$ complex (1.13 mg/mL = 0.015 mM) was photolyzed in the presence of 0.1 mM indole-3-propanol phosphate and 0, 0.007, 0.015, 0.029, 0.06, or 0.17 mM 6-azido-L-[^{14}C]tryptophan (2400 cpm/nmol) in a final volume of 0.2 mL (see Experimental Procedures). The amount of bound ^{14}C label, the residual activity, and protein concentration were determined on aliquots of the excluded volumes from centrifuged columns preequilibrated with 0.1 M potassium phosphate buffer, pH 7.0 (see Experimental Procedures). Since protein determined by absorbance at 278 nm (○) or folin (●) gave similar values, the bound ^{14}C label does not contribute significantly to the absorbance at 278 nm. No ^{14}C label was detected in the filtrates of a control reaction mixture containing 0.1 or 0.10 mM 6-azido-L-[^{14}C]tryptophan and no enzyme. Excluded volumes of similar control reaction mixtures containing 0.1 mM 6-azido-L-[^{14}C]tryptophan and either enzyme not photolyzed or enzyme added after photolysis contained less than 0.01 mol of ^{14}C label/mol of $\alpha\beta$.

Table III: Effect of Photolysis in the Presence of 6-Azido-L-tryptophan upon Activities and Absorbance of the $\alpha_2\beta_2$ Complex[a]

(A) Activities units/mg of $\alpha_2\beta_2$			
reaction	control	modified	%[b]
1	825	388	47
2	12	12	100
3	216	102	47

(B) Absorbance		ϵ (mM^{-1} cm^{-1})		
addition	λ_{max} (nm)	control	modified	%[b]
none	412	5.6	5.6	100
none	310	2.1	5.6	270
L-serine	412	−3.4 (Δ)	−1.2 (Δ)	35
L-serine + β-mercaptoethanol	468	31	11	33

[a] $\alpha_2\beta_2$ complex (0.05 mM) photolyzed for 2 min after each of three additions of 6-azido-L-tryptophan to a final concentration of 0.1 mM (modified) as described under Experimental Procedures had 24% of the activity in reaction 3 of the control that was photolyzed for 6 min in the absence of 6-azido-L-tryptophan. Each solution was supplemented with 0.1 mM pyridoxal phosphate and was dialyzed against 900 volumes of 0.1 M potassium phosphate, pH 7.8, containing 5 mM EDTA for 16 h. Enzyme solutions were assayed for activities in reactions 1–3 as described under Experimental Procedures. Absorption spectra were recorded on solutions of the dialyzed enzymes diluted to 0.01 mM in the presence and absence of 0.01 M L-serine or 0.01 M L-serine plus 0.05 M β-mercaptoethanol. The extinction coefficient per millimole of $\alpha\beta$ or change (Δ) at the indicated λ_{max} is given. [b] The activity or absorbance of the modified enzyme is expressed as a percent of the control.

^{14}C label that is retained in the β_2 subunit is similar to the stability at pH 7.0 of the ^{14}C label that is retained in the $\alpha_2\beta_2$ complex (Table II).

Activities of the Modified $\alpha_2\beta_2$ Complex. The activities

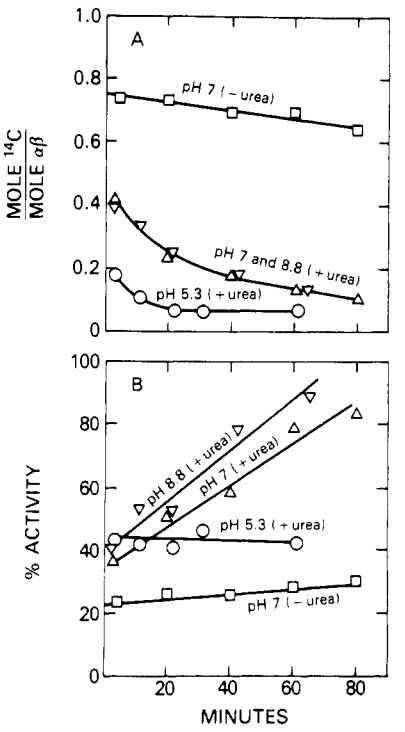


FIGURE 6: Effect of pH and of urea upon the stability of the incorporated photoaffinity label (A) and upon photoinactivation (B). Tryptophan synthase $\alpha_2\beta_2$ complex photolyzed 2 min after each of three additions of 6-azido-L-[^{14}C]tryptophan (2800 cpm/mmol) to a final concentration of 0.08 mM after each addition as described under Experimental Procedures had a residual activity 25% of that of a control solution photolyzed in the same way after each of three additions of water. The control and modified solutions (≃1 mg of $\alpha_2\beta_2$ in 1.0 mL) were applied at time = 0 to two 4-mL centrifuge columns equilibrated with one of the following buffers containing 0.01 M β-mercaptoethanol and 2 mM EDTA: 0.1 M potassium phosphate, pH 7.0 (□); 0.1 M potassium phosphate buffer, pH 7.0, containing 8 M urea (Δ); 0.1 M sodium acetate, pH 5.0, containing 8 M urea (○); 0.1 M Tris-HCl buffer, pH 9.0, containing 8 M urea (▽). The excluded volumes having measured pH values of 7.0, 7.0, 5.3, and 8.8, respectively, were incubated at 20 °C; 0.2-mL aliquots were removed at the indicated times and applied to 0.8-mL centrifuge columns. The filtrates were assayed for absorbance of 278 nm, activity, and radioactivity as described under Experimental Procedures. The results are plotted as moles of ^{14}C per moles of $\alpha\beta$ vs. time (A) and as percent activity of the modified enzyme relative to the activity of the control enzyme vs. time (B). Although the rates of control and modified solutions of $\alpha_2\beta_2$ complex in 8 M urea were linear within 1–2 min after dilution of 20 μL of enzyme solution into 1.0 mL of assay mixture at 37 °C containing excess α subunit (50 μg/1.0 mL), the specific activities of control enzymes after incubation in 8 M urea were only about half the specific activity of the enzyme prior to denaturation. A control experiment demonstrated that the $\alpha_2\beta_2$ complex was unfolded by passage through a centrifuge column equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 8 M urea, since the 8 mol of sulhydryl residues/mol of $\alpha\beta$ all reacted rapidly (within 2 min) with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

of the control and modified $\alpha_2\beta_2$ complexes are compared in Table IIIA. Whereas the modified enzyme has unchanged activity in reaction 2, the α reaction, it has reduced activity in reaction 1, the $\alpha_2\beta_2$ reaction, and in reaction 3, the β_2 reaction. Thus, the β_2 subunit, but not the α subunit, is inactivated by photolysis of 6-azido-L-tryptophan bound to the $\alpha_2\beta_2$ complex. This conclusion is supported by our finding (Table II) that ^{14}C label is incorporated into the β_2 subunit but not into the α subunit. We have also found that when the modified $\alpha_2\beta_2$ complex was resolved and separated into apo-β_2 and apo-α subunits by the method of Miles & Moriguchi (1977), the α subunit had normal activity in reaction 2 in the presence of unmodified β_2 subunit, whereas the β_2 subunit had

low activity in reaction 2 in the presence of unmodified α subunit (data not shown).

Spectral Properties of the Modified $\alpha_2\beta_2$ Complex in the Presence and Absence of Substrates. The absorption properties of dialyzed control and modified $\alpha_2\beta_2$ complexes are compared in Table IIIB. Although the two enzymes have identical absorption maxima at 412 nm, due to bound pyridoxal phosphate, the visible absorption spectrum of the modified enzyme is slightly shifted to lower wavelengths below 500 nm with a crossover point at 412 nm. The modified enzyme has a prominent shoulder at 310 nm. Addition of L-serine to the control $\alpha_2\beta_2$ complex results in a decrease in absorbance at 412 nm in the absence of β-mercaptoethanol and in a large increase in absorbance at 468 nm in the presence of β-mercaptoethanol (Miles et al., 1968; Goldberg et al., 1968; Miles, 1980). Since the absorbance changes observed with the modified enzyme (47% residual activity) were 33–35% of the change observed with the control enzyme, we conclude that the fraction of the enzyme that is inactivated does not form an enzyme–substrate complex that can be detected by absorbance changes.

Discussion

We have synthesized 6-azido-L-tryptophan from L-tryptophan by a novel procedure utilizing the 6-nitro derivative. Saito & Rilling (1981) have published an enzymatic procedure for preparation of 6-azido-L-tryptophan from 6-azidoindole and L-serine with the use of a crude preparation of tryptophan synthase from *Neurospora crassa*; however, the yields were low, and the product was impure. In our procedure, the use of the side chain protected 6-nitrotryptophan facilitates isolation and purification of the 6-azido-L-tryptophan product. The protecting groups are readily removed enzymatically in high yield under mild conditions. While this work was in progress, another method for the synthesis of 6-azido-L-tryptophan was published (Melhado & Leonard, 1983), without a requirement for side-chain protecting groups. The use of this compound as a photoaffinity reagent was not reported. The spectroscopic and physical properties of 6-azido-L-tryptophan prepared by our method are in good agreement with those reported by Melhado & Leonard (1983). While the latter authors state that diazotization of aminoindoles in hydrochloric acid gives low yields, in our hands the diazotization of the protected 6-aminotryptophan in dilute HCl (pH 2) gives yields comparable to those reported by Melhado & Leonard (1983) in aqueous 80% acetic acid. We have also prepared and isolated 6-azido-L-tryptophan (side-chain 1,2,3-^{14}C, labeled) in good yield from 6-azidoindole and uniformly labeled L-[^{14}C]serine with the homogeneous $\alpha_2\beta_2$ complex of tryptophan synthase from *E. coli*.

Our results demonstrate that 6-azido-L-tryptophan meets most of the criteria for an active site directed irreversible inhibition affinity label (Groman et al., 1977) and for a photoaffinity label (Chowdry & Westheimer, 1979; Baley & Knowles, 1977; Bayley, 1983): (1) 6-azido-L-tryptophan is a quasi-substrate for tryptophan synthase and is converted to a quinonoid intermediate, very similar to the intermediate formed with L-tryptophan (Figure 2A) [i.e., catalytic competence (Groman et al., 1977)]; (2) 6-azido-L-tryptophan binds reversibly and specifically to tryptophan synthase in the dark; binding is competitive with the substrate L-serine (Figure 2); (3) during illumination there is time- and concentration-dependent inactivation (Figures 3 and 4); (4) inactivation is prevented by the substrate L-serine as well as by the competitive inhibitors L-tryptophan, oxindolyl-L-alanine, and D-tryptophan (Table I); (5) inactivation is specific since the modified β_2 subunit still binds pyridoxal phosphate and α subunit and activates the α subunit in reaction 3 (Table IIIA); (6) inactivation is stoichiometric with incorporation of radiolabeled 6-azido-L-tryptophan (Figure 5).

We have taken several precautions and used several controls to try to avoid artifacts or nonspecific labeling that may occur in photoaffinity labeling experiments (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979; Bayley, 1983). Tris buffer and β-mercaptoethanol were used since they may act as scavengers of photogenerated intermediates at places other than the binding site. Sodium azide, which reacts with singlet oxygen, does not protect against photoinactivation. A short light path cuvette (0.2 cm) was used for photolysis so that the absorbance of the enzyme (10–50 μM) and added indole-3-propanol phosphate (100 μM) and 6-azido-L-tryptophan (100 μM) was about 0.4 at 280–300 μM and less than 1.3 at the highest concentration of protecting aromatic amino acid used (1 mM). Prephotolyzed 6-azido-L-tryptophan had no effect on activity (Table I) and did not bind to the $\alpha_2\beta_2$ complex (Figure 5 legend). Incubation with 6-azido-L-tryptophan in the "dark" during spectrophotometric titrations caused no irreversible inhibition of the $\alpha_2\beta_2$ complex. A non light absorbing substrate (L-serine) had a strong protective effect whereas a nonsubstrate (L-alanine) had no protective effect.

6-Azido-L-tryptophan is an excellent photoinactivator for the $\alpha_2\beta_2$ complex of tryptophan synthase since it binds tightly in the dark (the half-saturation value $[S]_{0.5}$ = 6.3 μM) and can thus be used at low concentrations (2–100 μM) comparable to the enzyme concentration (10–50 μM). At these low concentrations nonspecific reactions are less likely, and light can pass through the solution. Labeling by 6-azido-L-tryptophan is probably not quantitative because the enzyme-bound 6-azido-L-tryptophan partitions between breakdown and photoreaction with the enzyme during photolysis. Thus, photolysis of the $\alpha_2\beta_2$ complex nearly saturated with 6-azido-L-tryptophan results in complete loss of absorbance at 476 nm but in only 65% inactivation (Figure 4). Additional inactivation can be obtained by repeated addition of 6-azido-L-tryptophan and photolysis.

Since 6-azido-L-tryptophan forms a quinonoid intermediate with an absorption spectrum (Figure 2A) identical with that formed with L-tryptophan (Miles, 1980; Lane & Kirschner, 1981) and is bound somewhat more tightly than L-tryptophan in the presence of indole-3-propanol phosphate (Table I), the 6-azido group does not interfere with binding. This is not surprising since 6-azidoindole (Saito & Rilling, 1981) and 6-methylindole (Hall et al., 1962) are substrates for *Neurospora crassa* tryptophan synthase. Our finding that both formation of the quinonoid derivative in the dark and subsequent photoinactivation show saturation kinetics and the same concentration dependence (Figure 3A) is evidence that binding equilibrium is established before irradiation. The natural substrate, L-serine, competes with 6-azido-L-tryptophan for binding with a K_I close to its binding constant and protects the $\alpha_2\beta_2$ complex from photoinactivation. Indole-3-propanol phosphate, which binds to the active site of the α subunit, affects the binding of substrates and substrate analogues to the β_2 subunit in the $\alpha_2\beta_2$ complex indirectly by decreasing the binding constant values (Table I) and by increasing the steady-state level of the quinonoid intermediate formed with L-tryptophan (Miles, 1980; Lane & Kirschner, 1981) and with 6-azido-L-tryptophan (Figure 2A). Indole-3-propanol phosphate also increases the extent of photoinactivation of the $\alpha_2\beta_2$ complex by 6-azido-L-tryptophan (Table I, experiment 3 vs. experiment 4). Our finding that the extent of photoinactivation

is directly proportional to the absorbance at 476 nm of the quinonoid intermediate (Figure 3B) shows that photoinactivation results from photolysis of an intermediate bound at the active site of the β_2 subunit and suggests that the quinonoid intermediate may be a more effective photoinactivator than the other intermediates that accumulate in the absence of indole-3-propanol phosphate.

The modification produced by photolysis of 6-azido-L-tryptophan bound to the $\alpha_2\beta_2$ complex appears to be rather specific since it does not affect the activity of the α subunit in reaction 3 (Table IIIA) or the ability of the separated α subunit to stimulate the activity of unmodified β_2 subunit in reaction 2 (see Results). The modified $\alpha_2\beta_2$ complex binds pyridoxal phosphate but does not form complexes with L-serine or L-serine in the presence of β-mercaptoethanol, which can be detected spectrophotometrically.

6-Azido-L-tryptophan is a more specific photoaffinity reagent than 5-azidoindole, which labels several indole sites of the *E. coli* tryptophan synthase α subunit (Brock et al., 1983) and of the $\alpha_2\beta_2$ complex (Napier & Hardman, 1984). The reported protection by L-serine against photoinactivation of the $\alpha_2\beta_2$ complex by 5-azidoindole (Napier & Hardman, 1984) probably results from the conversion of 5-azidoindole to 5-azido-L-tryptophan followed by displacement of the 5-azido-L-tryptophan from its binding site by L-serine as reported here for 6-azido-L-tryptophan.

Initial experiments with unlabeled 6-azido-L-tryptophan indicated that photoinactivation might be partially reversible, since some reactivation occurred after dialysis of the modified enzyme for 16 h in the experiment described in Table III. Subsequent studies using 6-azido-L-[^{14}C]tryptophan showed that most of the ^{14}C label was slowly released at pH 7.0 ($t_{1/2} \simeq 24$ h) (Figure 6 and Table II). After heating of the $\alpha_2\beta_2$ complex to precipitate the α subunit by the method of Högberg-Raibaud & Goldberg (1977) (Table II), about half of the ^{14}C label remained attached to the β_2 subunit and not to the α subunit. The ^{14}C label attached to the β_2 subunit after this treatment was also slowly released. These results suggest that the incorporated ^{14}C label is either labile or bound tightly but not covalently. The photolysis products from some photoaffinity reagents may bind far more tightly than the ligand itself and may be removed only under denaturing conditions (Bayley, 1983). Since prephotolyzed 6-azido-L-tryptophan does not inhibit the $\alpha_2\beta_2$ complex (Table I) and does not bind to the $\alpha_2\beta_2$ complex (Figure 5 legend), the photoproduct is probably not tightly bound but is instead covalently bound.

Our observations that most of the ^{14}C label is released from the protein in 8 M urea rather rapidly at pH 7.0 and at pH 8.8 and even more rapidly at pH 5.3 (Figure 6A) also indicate that the main photoproduct of 6-azido-L-tryptophan is covalently attached but is labile. A noncovalent adduct would be expected to dissociate instantaneously from the denatured protein.

A small fraction (about 10%) of the incorporated label remains attached after treatment of the $\alpha_2\beta_2$ complex with 8 M urea at pH 5.3 for 60 min or after precipitation of the $\alpha_2\beta_2$ complex with acid (Table II). These results indicate that photoinactivation of tryptophan synthase by 6-azido-L-tryptophan results from at least two different classes of covalent adducts, of which one is stable and one is labile.

Arylnitrenes have been proposed to react with nucleophilic groups on proteins to form several more or less labile products (Bayley, 1983; Bayley & Knowles, 1977). If the nucleophile is an amino or thiol group, the product is a stable arylamine or aryl thioether (Scriven et al., 1979; Bayley, 1983). However, when the attacking nucleophile is a carboxylate, the product is an extremely unstable carboxyazepine (Colman et al., 1981). The instability of the major adduct generated in our experiments leads us to conclude that a carboxylate or carboxamide group in the active site is being modified. Thus, our data provide the first implication of a catalytically essential carboxylate in the active site of the β_2 subunit of tryptophan synthase. The second site that is labeled in a stable way is probably one or more sulfhydryl or lysyl residues near the 6-azido-L-tryptophan binding site. Although the amount of photoproduct incorporated into this site is low, it might be possible to identify this site by using 6-azido-L-[^{14}C]tryptophan with a high specific activity.

6-Azido-L-tryptophan is a potentially useful photoaffinity label for other proteins that bind tryptophan. These include anthranilate synthase, tryptophanase, the aroH isozyme of *E. coli* 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate synthase, tryptophanyl-t-RNA synthetase, the tryptophan repressor, monoamine oxidase, tryptophan 2,3-dioxygenase, indoleamine 2,3-dioxygenase, and tryptophan 5-hydroxylase.

Registry No. I, 73-22-3; II, 46885-76-1; III, 97072-23-6; IV, 97072-24-7; V, 97072-25-8; VI, 97072-26-9; VII, 97072-27-0; VIII, 81524-70-1; L-serine, 56-45-1; D-tryptophan, 153-94-6; oxindolyl-L-alanine, 32999-55-6; tryptophan synthase, 9014-52-2.

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