EFFECTS OF MODIFICATION OF THE β_2 SUBUNIT AND OF THE $\alpha_2\beta_2$ COMPLEX OF TRYPTOPHAN SYNTHASE BY α-CYANOGLYCINE, A SUBSTRATE ANALOG

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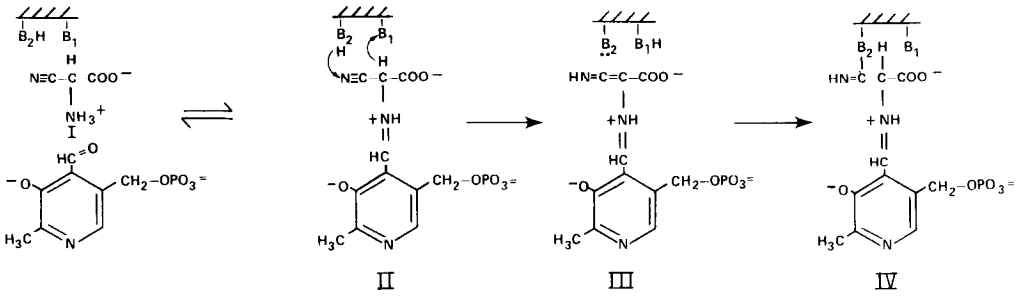
α-Cyanoglycine inactivates the pyridoxal-P forms of the β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase; an intense chromophore at 430 nm forms concomitantly. The slow reactivation of the modified β_2 subunit upon dialysis ($t_{1/2}$ = 24 hours) is prevented by addition of α subunit, which presumably acts by changing the environment of the chromophoric derivative. These data and the observed protection from inhibition by L-serine indicate that α-cyanoglycine acts as a substrate analog which undergoes a second, largely irreversible reaction at the active site of the β_2 subunit. Modification of the β_2 subunit increases its affinity for the α subunit. Modification of the $\alpha_2\beta_2$ complex increases its stability to heat, urea, and low pH.

A class of highly specific, irreversible enzyme inhibitors was first discovered by Bloch and coworkers (1) and has recently been reviewed by Rando (2). These inhibitors are relatively unreactive chemically until they have been converted to reactive intermediates by their target enzymes. Abeles has recently proposed that α-cyanoglycine may be such an inhibitor and has found that it inhibits certain pyridoxal-P dependent enzymes.[1] A possible mechanism for the inhibition of pyridoxal-P enzymes by α-cyanoglycine is shown in Scheme I. α-Cyanoglycine (I) first forms a Schiff base with enzyme-bound pyridoxal-P (II). Removal of the α-proton of α-cyanoglycine by a nucleophilic group on the enzyme (B_1) is followed by the rearrangement of the product to yield a nitrogen analog of ketene (III). This highly reactive intermediate may react with a nucleophilic group on the enzyme (B_2) to produce an inactive derivative of the enzyme (IV). In this report α-cyanoglycine is tested on both the β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli*. The β_2 subunit, which binds 2 moles of pyridoxal-P (PLP) per mole of enzyme dimer, catalyzes Reactions 1 and 4

[1] Personal communication of Dr. Robert H. Abeles.



SCHEME I

(3). The α subunit alone has a small activity in Reaction 2 (3). The $\alpha_2\beta_2$ complex catalyzes Reactions 1, 2, and 3 (3).

$$\text{Indole} + \text{L-Serine} \xrightarrow[\beta_2,\ \alpha_2\beta_2]{\text{PLP}} \text{L-Tryptophan} + H_2O \quad (1)$$

$$\text{Indole-3-glycerol-phosphate} \underset{\alpha,\ \alpha_2\beta_2}{\rightleftharpoons} \text{Indole} + \text{D-Glyceraldehyde-3-phosphate} \quad (2)$$

$$\text{Indole-3-glycerol-phosphate} + \text{L-Serine} \xrightarrow[\alpha_2\beta_2]{\text{PLP}} \text{L-Tryptophan} + \text{D-Glyceraldehyde-3-phosphate} \quad (3)$$

$$\text{L-Serine} \xrightarrow[\beta_2]{\text{PLP}} \text{Pyruvate} + NH_3 \quad (4)$$

MATERIALS AND METHODS

α-Cyanoglycine was a generous gift of Dr. Robert H. Abeles and was synthesized by the method of Ressler *et al.* (4). The crystalline α and β_2 subunits and $\alpha_2\beta_2$ complex of tryptophan synthase were prepared and assayed in Reaction 1 as described previously (5-7). Reaction 2 was assayed in the presence of 1 M hydroxylamine (7) or spectrophotometrically (8). Reaction 4 was also assayed spectrophotometrically (9). Indole-3-glycerol-phosphate wwas synthesized enzymatically (10).

RESULTS AND DISCUSSION

Fig. 1A shows the effects of treatment of the holo β_2 subunit and of the holo $\alpha_2\beta_2$ complex with 1 mM α-cyanoglycine. Both enzymes showed a rapid increase in absorbance at 430 nm which was concomitant with decrease in activity. Treatment of the apo β_2 subunit and apo $\alpha_2\beta_2$ complex under similar conditions

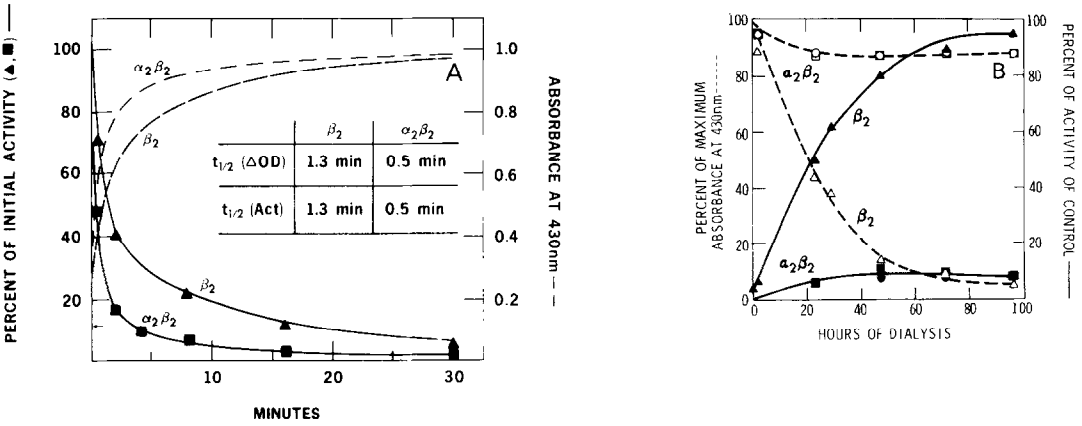


FIG. 1. *Modification of the holo β_2 subunit and holo $\alpha_2\beta_2$ complex with α-cyanoglycine: effects of time of modification and of subsequent dialysis on the absorbance at 430 nm and activities.* Holo β_2 subunit (1.45 mg/ml in 0.1 M potassium phosphate, pH 7.8, containing 0.2 mM dithiothreitol, 5 mM EDTA, and 0.02 mM pyridoxal-P) and $\alpha_2\beta_2$ complex containing the same concentration of holo β_2 subunit plus 0.65 mg of α subunit in the same buffer, were treated with 1 mM α-cyanoglycine at 22°. A. Absorbance at 430 nm was measured continuously for $\alpha_2\beta_2$ complex and β_2 subunit (-----); the initial absorbance at 430 nm is indicated by an arrow. The serine deaminase activity of the β_2 subunit (▲) and the tryptophan synthase activity of the $\alpha_2\beta_2$ complex (■) were measured at intervals by dilution of aliquots directly into the appropriate reaction mixtures for assay. The concentration of free α-cyanoglycine added to the reaction mixture was not inhibitory. B. Solutions of α-cyanoglycine treated β_2 subunit (Δ), and $\alpha_2\beta_2$ complex (□), and a solution of treated β_2 subunit to which 0.65 mg α subunit was added after treatment for 30 minutes (○), were dialyzed against 0.1 M potassium phosphate, pH 7.8, containing 0.01 M β-mercaptoethanol. Activities (see A) expressed as a percent of untreated controls which were dialyzed similarly, are shown by closed symbols and solid curves. Absorbance at 430 nm is shown by open symbols and dashed lines.

in the absence of pyridoxal-P resulted in the loss of less than 10% activity in 60 minutes; activity was measured in the presence of pyridoxal-P after dilution of both enzyme and α-cyanoglycine to give a concentration of α-cyanoglycine which was not inhibitory. When the holo $\alpha_2\beta_2$ complex was treated with 1 mM α-cyanoglycine in the presence of 0.1 M L-serine for 20 minutes before assay, the loss of activity was only 6%.

The findings that inhibition by α-cyanoglycine is dependent on the presence of pyridoxal-P and is prevented by L-serine suggest that α-cyanoglycine acts like a substrate or substrate analog and forms a Schiff base intermediate of type II (Scheme I) with enzyme-bound pyridoxal-P. However, the rate of inhi-

bition by α-cyanoglycine ($t_{\frac{1}{2}}$ = 0.5 minute for the $\alpha_2\beta_2$ complex or 1.3 minutes for the β_2 subunit) is much slower than would be expected for Schiff base formation; the $\alpha_2\beta_2$ complex forms a Schiff base intermediate with L-serine within the dead-time of a stopped flow instrument (11). The relatively slow rate of inhibition and the finding (Fig. 1B) that the inhibition and the absorbance of the modified β_2 subunit are only slowly decreased upon dialysis ($t_{\frac{1}{2}}$ = 24 hours) suggest that inhibition by α-cyanoglycine probably results from a second slower step to form a more stable derivative such as III or, more probably, IV (Scheme I). Treatment of modified β_2 subunit or $\alpha_2\beta_2$ complex with phenylhydrazine under acid conditions (12) yielded 2 moles of pyridoxal-P phenylhydrazone per mole of β_2 subunit. This would be the expected result if the modified enzyme had structures II, III, or IV, but not if it were a ketimine derivative.

Addition of α subunit to the β_2 subunit either before or after treatment with α-cyanoglycine largely prevented reactivation upon dialysis (Fig. 1B). This suggests that the chromophoric derivative is buried in a hydrophobic environment in the $\alpha_2\beta_2$ complex which protects it from hydrolysis or from removal by dialysis.

Table I compares the activities of the holo $\alpha_2\beta_2$ complex and the holo $\alpha_2\beta_2$ complex modified by α-cyanoglycine or reduced with $NaBH_4$ in Reactions 1 and 2 and the effect of excess unmodified β_2 subunit on each reaction. Both of the modified complexes had much lower activities than the untreated $\alpha_2\beta_2$ complex in Reaction 1, which is catalyzed by the active site of the β_2 subunit, but had higher activities than the untreated $\alpha_2\beta_2$ complex in Reaction 2, which is catalyzed by the active site of the α subunit. Excess unmodified β_2 subunit stimulated both of the activities of the untreated $\alpha_2\beta_2$ complex by producing saturation of the free α subunit which is present in equilibrium with active $\alpha_2\beta_2$ complex at the enzyme concentration used for assay (see Fig. 2). Excess unmodified β_2 subunit produced a much larger stimulation of the low activity of the borohydride reduced $\alpha_2\beta_2$ complex in Reaction 1, presumably by exchanging with the reduced β_2 subunit to give native $\alpha_2\beta_2$ complex and free reduced β_2

TABLE I

EFFECT OF α-CYANOGLYCINE MODIFICATION AND $NaBH_4$ REDUCTION OF THE $\alpha_2\beta_2$ COMPLEX ON ACTIVITIES ASSAYED IN THE PRESENCE AND ABSENCE OF EXCESS UNTREATED β_2 SUBUNIT

Treatment of $\alpha_2\beta_2$ complex [a]	Activity in Reaction 1 [b]		Activity in Reaction 2 [b]	
	$\alpha_2\beta_2$ alone	+ excess β_2	$\alpha_2\beta_2$ alone	+ excess β_2
	units/mg $\alpha_2\beta_2$			
None	1,120	1,350	320	505
$NaBH_4$	200	800	700	885
α-Cyanoglycine .	274	223	700	700

[a] $\alpha_2\beta_2$ complex (2.4 mg/ml in 0.1 M potassium phosphate, pH 7.8, containing 0.01 M β-mercaptoethanol and 0.04 mM pyridoxal-P) was treated with 1 mM α-cyanoglycine for 30 minutes or with 1 mM $NaBH_4$ and then dialyzed against the above buffer.

[b] Aliquots of $\alpha_2\beta_2$ complex were assayed for activity in Reaction 1 or in Reaction 2 (in the presence of hydroxylamine) in the presence or absence of a 10 X molar excess of β_2 subunit. Assay mixtures were preincubated for 30 minutes at 4° before addition of L-serine or indole-glycerol-P. Results are corrected for the activity in Reaction 1 due to the excess β_2 alone. A control experiment showed that the absorbance at 430 nm of the α-cyanoglycine treated $\alpha_2\beta_2$ complex was unaffected by incubation with 1 M NH_2OH.

subunit. In contrast, added β_2 subunit did not stimulate the activity of the α-cyanoglycine modified $\alpha_2\beta_2$ complex in Reaction 1. This failure of the α-cyanoglycine modified $\alpha_2\beta_2$ complex to undergo subunit interchange is probably due to the increased affinity of the subunits which is shown in Fig. 2; α subunit has an 8 times higher affinity for the modified β_2 subunit (apparent association constant "K_A" = 1.5×10^8 M^{-1}) than for the untreated β_2 subunit ("K_A" = 1.8×10^7 M^{-1}). A similar enhancement of the apparent association constant by L-serine has been previously observed (8).

The increased affinity of the α and the modified β_2 subunits is probably responsible for the increased stability of the modified $\alpha_2\beta_2$ complex to conditions of heat (A), urea (B), and low pH (C) (Fig. 3). Loss of activity of

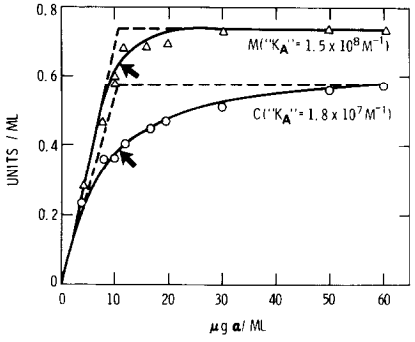


FIG. 2. *Saturation of the untreated and α-cyanoglycine modified β_2 subunit by α subunit in Reaction 2.* The activities of holo β_2 subunit (C) and holo β_2 subunit treated with 1 mM α-cyanoglycine (M) as described in Fig. 1 were measured in Reaction 2 by the spectrophotometric assay (8) in the presence of 0.1 mM pyridoxal-P and the indicated concentration of α subunit. The final concentration of control or modified β_2 subunit was 25 μg/ml which is 5 x 10^{-7} M in β chains. The solid lines are drawn through the experimental data points (Δ) for M and (O) for C. The dashed lines drawn by extrapolation of the linear portions of each curve show a titration curve which would be obtained if no dissociation of the $\alpha_2\beta_2$ complex occurred. The intersection point of the dashed lines gives the concentration of α which forms an active 1 : 1 complex with the added β. This mixture of equimolar α and β chains will be called an equivalent mixture.* The difference between the dashed and solid curves indicates that dissociation does occur and has been used to determine an apparent association constants "K_A" for this dissociation where (αβ) is the concentration of the complex and (α) and (β) are concentrations of the free α and β subunits and α_{total} = (α) + (αβ) (8).

$$\alpha + \beta \rightleftharpoons \alpha\beta; \quad "K_A" = (\alpha\beta) / (\alpha)(\beta).$$

"K_A" has been calculated from the observed data for the equivalent mixture where $(\alpha_{total}) = (\beta_{total})$; the fraction of the α and β chains present as active complex is determined from the ratio of the observed activity of the equivalent mixture (indicated by an arrow) to the activity of the same concentration of β chain saturated by α chains (indicated by the horizontal, dashed line). Then

$$(\alpha\beta) = (\alpha_{total}) \left(\frac{\text{activity of equivalent mixture}}{\text{activity in presence of excess } \alpha}\right)$$

$$\text{and } (\alpha) = (\beta) = (\alpha_{total}) - (\alpha\beta).$$

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* The observed concentration of α subunit in the equivalent mixture (10.5 μg/ml or 3.6 x 10^{-7} M in α chains) is lower than the concentration of β chains added (5 x 10^{-7} M) because of some inactive β chains. The concentration of active β chains in the equivalent mixture has been taken to be 3.6 x 10^{-7} M in calculation of "K_A."

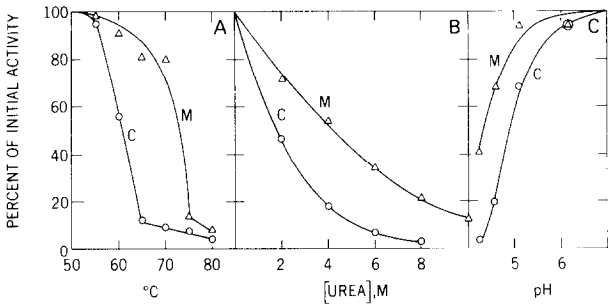

FIG. 3. *Effect of modification of the $\alpha_2\beta_2$ complex with α-cyanoglycine on its stability to heat, urea, and low pH.* $\alpha_2\beta_2$ complex (2.6 mg/ml in 0.1 M potassium phosphate, pH 7.8, containing 0.01 M β-mercaptoethanol and 0.04 mM pyridoxal-P) treated for 30 minutes with 1 mM α-cyanoglycine and dialyzed against the above buffer (M) and untreated control $\alpha_2\beta_2$ complex (C) were used for these experiments. A. Aliquots (0.1 ml) of control and modified $\alpha_2\beta_2$ complex were heated for 3 minutes at each of the indicated temperatures, chilled, and centrifuged. Aliquots were assayed for activity in Reaction 2 in the presence of hydroxylamine (7). B. Aliquots of control and modified $\alpha_2\beta_2$ complex were assayed for activity in Reaction 2 in the presence of 1 M NH_2OH and the indicated concentrations of urea. C. Aliquots of control and modified $\alpha_2\beta_2$ complex were diluted 1 : 10 with 0.1 M sodium acetate buffers at pH 4 and 5 and with 0.1 M potassium phosphate buffers at pH 6 and 7. The actual pH was determined. Each solution was incubated 30 minutes at 4° before assay in Reaction 2 in the presence of hydroxylamine (7). The pH of each assay was 7.0 and was not changed by the addition of a small aliquot of the enzyme solution.

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the modified $\alpha_2\beta_2$ complex at high temperature and low pH was accompanied by disappearance of the absorbance at 430 nm and precipitation of denatured protein. Urea had no effect on the chromophore at 430 nm at concentrations lower than 8 M; at this concentration the chromophore disappeared slowly ($t_{1/2}$ = 30 minutes) and the activity of the $\alpha_2\beta_2$ complex in Reaction 1 was fully restored after 2 hours. The activity was measured after dilution of the enzyme 10-fold with 0.1 M potassium phosphate, pH 7.8, containing 0.1 M β-mercaptoethanol and 0.1 mM pyridoxal-P to renature the proteins (13). Thus, denaturation of the $\alpha_2\beta_2$ complex results in release of the chromophoric derivative and in restoration of enzyme activity under conditions where renaturation is possible. Although the relative stability of the α-cyanoglycine derivative suggests that it is attached covalently to the β_2 subunit (IV, Scheme I), the findings that inhibition can be reversed by extensive dialysis or by denaturation indicate that any covalent link is rather labile.

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