Carbamylation of Aspartate Transaminase and the pK Value of the Active Site Lysyl Residue*

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Abnormal lysyl residues can be detected in aspartate transaminase by following the rate of reaction of amino groups with KCN and the rate of enzymatic inactivation. Peptide isolation subsequent to carbamylation of the apoenzyme produces a peptide which is absent in the carbamylated holoenzyme. The composition of the carbamylated peptide matches that of a tryptic peptide containing the active site Lys-258.

The holoenzyme retains full catalytic activity after carbamylation of its NH2-terminal alanine and lysyl residues other than Lys-258, which is protected by aldimine formation with pyridoxal phosphate. Apoenzyme prepared from KCN-treated holoenzyme (apoenzyme') is susceptible to further carbamylation at Lys-258 with irreversible loss of catalytic activity. Carbamylation of the active site lysyl residue is 25 to 50 times more rapid than that of the other 18 lysyl residues of aspartate transaminase.

The kinetics of inactivation by KCN at different pH values served to determine the pH-independent second order rate constant (k) and the pK of the amino group of Lys-258. These values are pK = 7.98 ± 0.08 and k = 146 ± 5 M⁻¹s⁻¹, which are similar to the values determined for carbamylation of the NH₂-terminal groups of human hemoglobin (Garner, M. H., Bogardt, R. A., and Gurd, E. R. N. (1975) J. Biol. Chem. 250, 4398-4404). The pK value for Lys-258 is as low as that for a group in the active site region which can perturb a 19F nuclear magnetic resonance probe inserted into that region (Martinez-Carrion, M., Slebe, J. C., Boettcher, B., and Relimpio, A. M. (1976) J. Biol. Chem. 251, 1853-1858).

Apoenzyme carbamylated at Lys-258 can accept pyridoxal phosphate at the active site even though no Schiff base is formed. Furthermore, this active site carbamylated holoenzyme will form spectroscopically detectable enzyme-substrate complexes with amino acids. The complexes slowly convert to species with absorbance identical with that of enzyme in the pyridoxamine phosphate form.

The determination of the pK values of amino acid residues at the active center of enzymes in solution is a complex task. The methodology is largely limited to spectroscopic probing for the determination of an individual pK for those few cases where a distinctive spectroscopic property can be detected in the amino acid residue under investigation (1-3) or in kinetic procedures which follow the pK dependence of the reaction rate of a chemical modification for a specific residue in the enzyme (4-9).

Lysyl residues in peptides show average pK values of ~10. Several proteins, however, have shown unusually reactive lysyl residues with anomalous pK values which can be as low as 5.9 for oxalacetate decarboxylase (4) and 7.70 ± 0.3 in glutamate dehydrogenase (7).

The unusual reactivity of some lysyl residues in proteins may manifest itself in their extraordinary reactivity with pyridoxal-P° with which they form Schiff bases that can be easily reduced and radioactively labeled (9-12). This use of pyridoxal-P as a marker of specific regions of a protein in solution or of membranes is increasingly gaining acceptance as a tool for specific tagging of proteins. On the other hand, it now appears that pyridoxal-P may not be a specific reagent for lysyl residues only, since NH₂-terminal groups in some proteins may also be susceptible to Schiff base formation with pyridoxal-P (13).

The family of the pyridoxal-P-dependent enzymes contains an unusually reactive lysyl residue which, with the exception of that in glycogen phosphorylase, is bound to the active site chromophore, pyridoxal-P, as an internal aldimine (14). The determination of the pK value of the unusual amine, which may be a prototype for the behavior of other less discriminating lysyl residues, has remained elusive. Only recently we have detected a group in the pyridoxal-P-binding region of aspartate

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The abbreviations used are: pyridoxal-P, pyridoxal phosphate; pyridoxamine-P, pyridoxamine phosphate; NMR, nuclear magnetic resonance.
transaminase which ionizes with a pK value of 8.3 ± 0.1 (15). The NMR spectroscopic evidence obtained with a 143-marked analogue of pyridoxal-P on which this pK value is based does not permit us to assign this low pK value specifically to Lys-258, the active site lysyl residue. Among other possibilities, the NH2-terminal group of the protein should possess a pK value near 8.0 and the likelihood of it affecting the spectroscopic probe at the active site cannot be eliminated. The two possibilities should be distinguishable by the use of a proper chemical kinetic mean which makes use of the preliminary estimates of the pK value of the ionizing group at the active site region to devise methods for the selective modification of unprotonated amines with cyanate.

In this work we introduced, separately, the cyanate label in the NH2-terminal group and in Lys-258. From the dependence of the rate of carbamylation as a function of pH, the pK value of the active site lysyl residue in the apoprotein was evaluated.

EXPERIMENTAL PROCEDURE

Materials

Malate dehydrogenase, NADH, L-cysteine sulfonic acid, pyridoxal phosphate, L-aspartate, and α-ketoglutarate were obtained from Sigma Chemical Co. Potassium cyanate was purchased from J T Baker Co., potassium 143-cyanate, 56 mCi/mmol, from Amersham/Searle, and l-tosylamido-2-phenylethyl chloromethyl ketone trypsin was acquired from Worthington, glutamic acid from Calbiochem, and diethylaminoethyl cellulose, DE32, from Whatman.

Aspartate Transaminase

Cytoplasmic aspartate transaminase (EC 2.6.1.1) was isolated from pig heart in the pyridoxal-P holoenzyme form, and apoenzyme was prepared as previously described (16). Protein concentrations were calculated from the optical densities at 280 nm where the extinction coefficient is 140,000 for a molecular weight of 94,000 (17).

Spectrophotometric Measurements

The absorption spectra were obtained with 1-cm light path quartz cells in a Cary model 15 recording spectrophotometer equipped with a 0 to 0.1 absorbance unit expanded scale slide wire at 25°. Additions were made with micropipettes, and the reference samples contained all components except the enzyme.

Aspartate transaminase activity was measured spectrophotometrically by following the NADH formation in a total volume of 1 ml: excess malate dehydrogenase, 0.2 mM NADH, 6 mM α-ketoglutarate, 100 mM l-aspartate, and 50 mM sodium phosphate buffer (pH 7.5). One unit of activity was defined as the number of micromoles of NADH oxidized per min per mg of enzyme. All experimental results are the average of at least two determinations.

Inactivation of Aspartate Transaminase

The cyanate inactivation was studied by adding an appropriate amount of freshly prepared potassium cyanate stock solution (0.4 M) to enzyme solutions (5 to 100 mg/ml) at 37° in 0.05 M sodium phosphate buffer, pH 7.4, unless stated otherwise. In most of the inactivation experiments a final concentration of 0.2 M KCNO was used. However, in the studies of the rate of inactivation as a function of cyanate concentration, the KCNO concentration ranged from 0.025 M to 0.2 M in 0.05 M boric acid/borax buffer, pH 7.6. The ionic strength, in this case, was maintained constant by the addition of KCl. At specified times, aliquots (5 to 10 μl) were withdrawn and diluted to 1 ml with 0.1 M Tris-chloride buffer, pH 8.0, and were assayed for activity. The cyanate concentration was determined at 80% efficiency for 143. From the measured specific activity of the [143]cyanate solution in the presence of transaminase the moles of KCNO incorporated in each mole of enzyme subunit were calculated. After 32 min of incubation only about 10% of the activity remained and most of the reaction mixture (1.8 ml) was transferred into a Sephadex G-25 column (2 x 30 cm) and equilibrated with 0.1 M Tris-chloride buffer, pH 8.0. The remaining sample was left for further reaction for the longer time periods.

Labeling of Enzyme

Holo- and apotransaminases were prepared by 30-min incubation of the protein with 143-cyanate and the excess of reagent was removed by filtration as described above. Apotransaminase* (apoenzyme) was prepared from holoenzyme by the following pretreatment: holoenzyme was dialyzed exhaustively against deionized water and reacted with iodoacetamide (0.4 M) without loss of activity. The alkylated holoenzyme was then incubated with 0.2 M KCNO for 2 h and applied to the Sephadex G-25 column. After this treatment apotransaminase* (apoenzyme) as described above for holo- and apotransaminase. The radioactive material from holo-, apo-, or apoenzyme* was collected from a Sephadex G-25 column and concentrated by vacuum dialysis. A sample (40 to 60 mg) was incubated overnight with 6 mM guanidine hydrochloride containing 0.1 M Tris-chloride buffer, pH 8.0, 1 mM diithiothreitol, and 10 mM EDTA at 25° and then carbamylated by adding 0.4 ml of 0.5 M sodium iodoacetate, with incubation for 2 h at room temperature.

Hydrolysis by Trypsin

Trypsin digestion of both apoenzyme and apoenzyme* was conducted separately. The carbamylated, carboxymethylated preparations were dialyzed overnight against deionized water at 5°. The precipitates were collected by centrifugation, suspended in 10 to 15 ml of 0.05 M N-ethylmorpholine acetate buffer, pH 6.0, and hydrolyzed with TFA-trypsin (0.8 mg) for 12 h at 25°.

Isolation of Radioactive Peptide

Sephadex G-50 Gel Filtration—The tryptic digest was concentrated by rotary evaporation, dissolved in 9 ml of 0.05 M N-ethylmorpholine acetate buffer, pH 8.0, and passed through a Sephadex G-50 column (1.6 x 56 cm) which was previously equilibrated with the same buffer. Peptides were eluted with the same solvent at a flow rate of 40 ml/h. Eluted fractions (2.2 ml) were monitored spectrophotometrically at 280 nm and those fractions containing radioactive peptides were combined and concentrated. The column was exhaustively washed with the buffer between runs.

Fractionation by DEAE-Cellulose Chromatography—Labeled tryptic peptides were dissolved in 2 ml of 20 mM Tris-chloride buffer, pH 8.0, and 1 mM EDTA and adsorbed onto a DEAE column (0.9 x 12 cm) equilibrated with the same buffer. Fractions of 1.75 ml were collected at a flow of 20 ml/h. Elution was performed by applying first a linear

\[
\frac{C_a}{k_{obs}} = \frac{K_a}{k} + \left(\frac{H^+}{k}\right)^2 + \frac{K_a + K_b}{k_{obs}}(K_a + K_b)
\]
gradient from 0 to 0.1 M NaCl, followed by another gradient from 0.1 to 0.5 M NaCl (50 ml each) in Tris-chloride buffer, pH 8.0, and 1 mM EDTA.

**Sephadex G-10 Gel Filtration and Paper Chromatography**—Fractions (1.75 ml) from the last peak of the DE32 run were combined, concentrated, and chromatographed on a Sephadex G-10 column (0.9 × 70 cm) equilibrated with 0.05 M N-ethylmorpholine acetate buffer, pH 8.0, at a flow rate of 6 ml/h. The radioactive fractions were pooled and concentrated. The labeled active site peptide was subjected to an ascending paper chromatography with the mixture 1-butanol/glacial acetic acid/water (120/30/50) for 18 h. The portions with radioactivity were eluted from the paper with 0.05 M N-ethylmorpholine acetate buffer, pH 8.0, concentrated by rotary evaporation, and the buffer was eliminated with repetitive dilutions and evaporations.

**Amino Acid Analysis of Peptide**

NH₂-terminal residue determination was carried out according to the procedure described by Stark and Smyth (19). Hydantoin hydrolysis was performed for 20 h in 0.2 M NaOH at 110°C in a sealed tube and the amount of alanine was determined on the Beckman model 117 automatic amino acid analyzer. For the cyclization to hydantoin, 0.41 pmol of carbamyl labeled active site peptide was subjected to an overall second order reaction (Fig. 2). Under the same experimental conditions no activity losses are detected when either apoenzyme is incubated in the absence of KNCO.

**Radioactivity Assays**

Millipore filters and aliquots (25 to 50 µl) of column eluate fractions were added to 10 ml of scintillation fluid in vials and counted with a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid was obtained by mixing 1 liter of toluene (scintillation grade), 8 g of 2,5-diphenyloxazol, 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (scintillation grade), and 500 ml of Triton X-100; vials were routinely counted for 2 min. The paper chromatogram radioactivity was measured on a Packard model 7201 radiochromatogram scanner.

**RESULTS**

**Inactivation of Pig Heart Supernatant Aspartate Transaminase by Cyanate**—The susceptibility of the aspartate transaminase to inactivation by reaction with 0.2 mM KNCO depends on the presence or absence of co-enzyme. Holoenzyme with the active site lysyl residue blocked by pyridoxal-P, as an internal aldimine, is functionally unaffected by the cyanate treatment. On the other hand, the same cyante treated holoenzyme, after conversion to its apoenzyme form (apoenzyme') can be subjected to the same cyanate treatment with a resulting rapid and total inactivation with a pseudo-first order rate constant of 0.09 min⁻¹ at 37°C (Fig. 1). The same rate of inactivation is observed on cyanate treatment of apoenzyme produced from native, untreated holoenzyme. No recovery of activity can be detected by extensive dialysis or passage through a Sephadex G-25 column for either apoenzyme. Incubation of the cyanate-treated apoenzymes with 1 × 10⁻⁴ M pyridoxal-P is also ineffective in restoring catalytic activity.

The rates of inactivation by cyanate treatment, at constant pH and enzyme concentration, are dependent on the cyanate concentrations. Plots of log of activity versus time of cyanate treatment are linear, indicating their first order dependence. The pseudo-first order rate constant observed (kₗₒₑₜ) follows a linear relationship with cyanate concentration characteristic of an overall second order reaction (Fig. 2). Under the same conditions no activity losses are detected when either apoenzyme is incubated in the absence of KNCO.

**Incorporation of [¹⁴C]Cyanate to Apo- and Holoenzyme**—The pattern of incorporation of [¹⁴C]cyanate into transaminase depends on the form of enzyme used (Fig. 3). In all cases, holo- and apoenzymes, we can distinguish at least two phases representing groups of residues with diverse susceptibility to cyanate. At pH 7.4 most of the fast-reacting groups react within the first 20 min of treatment with 0.2 mM KN¹⁴CO. In the apoenzyme the number of groups modified in the initial phase is less than 3/subunit. In the holoenzyme the fast phase corresponds to incorporation into 1 residue. However, in apoenzyme', produced from extensively cyanate-treated holoenzyme, the same biphasic behavior is observed (Line 2, Fig. 3) with incorporation of radioactivity in 1 residue/subunit during

**FIG. 1. Effect of carbamylation on the activity of aspartate transaminase.** 1, Holoenzyme; 2, apoenzyme' (Δ); and apoenzyme (Δ). Enzyme (final concentration 1.5 to 2.0 mM) at pH 7.4 and 37°C.

**FIG. 2.** Left, pseudo-first order plots for the inactivation of apo-transaminase (4.86 × 10⁻⁷ M) by cyanate at pH 7.6 and 37°C. Numbers represent molar concentration of KNCO. Right, pseudo-first order rate constants of inactivation of apoenzyme plotted as a function of cyanate concentration.
The pK of Lys-258 in Aspartate Transaminase

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Fig. 3. Carbamylation of the α and ε NH₂ groups of 1, apoenzyme; 2, apoenzyme*; and 3, holoenzyme. (For details see "Experimental Procedure"). Arrow, radioactivity incorporated measured after passage of sample through a Sephadex G-25 column (2 x 30 cm).

Table I

| Enzyme form | [³¹C]KNCO treatment | Incorporation | Activity | End group |
|-------------|----------------------|---------------|----------|-----------|
|             | min                  | μmol [³¹C]/μmol monomer | % | μmol alanine/μmol monomer |
| Holo        | 32                   | 1.6           | 100      |           |
|             | 190                  | 9.4           | 100      | 1.00b     |
| Apo         | 32                   | 3.8           | 10       |           |
|             | 120                  | 5.2           | 5        | 1.00      |
| Apo*        | 32                   | 2.0           | 10       | 1.00      |
|             | 120                  | 3.8           | 5        |           |

* Conditions as in Figs. 1 and 3.

b [³¹C]-labeled compound.

d The fast phase. The cyanate is always incorporated by carbamylation of amino groups. The groups modified are the NH₂-terminal alanine residue, identified after preparation and hydrolysis of its hydantoin derivative, and the ε-amino groups of lysyl residues (Table I). After KN[¹⁴CO] treatment of apoenzyme and holoenzyme the carbamylated NH₂-terminal residue contains radioactivity but it is absent in [¹⁴C]-carbamylated apoenzyme*. None of the radioactivity is removed by dialysis or passage through the Sephadex G-25 column and the values of [¹⁴C] are identical whether measured after trichloroacetic acid precipitation of the protein or passage through the Sephadex column.

The initial phase of holoenzyme cyanate incorporation represents carbamylation of the NH₂-terminal alanine. The rest of the modification can be accounted for by the appearance of radioactive homocitrulline in the chromatography of acid hydrolysates (Fig. 4). The same amino acids are modified during [¹⁴C] carbamylation of apoenzyme except for the total greater [¹⁴C] incorporation during the fast phase which is to be expected if unusual lysyl groups become available after removal of pyridoxal-P. In apoenzyme* the only amino acid radioactively modified appears to be lysine detected as [¹⁴C]-labeled homocitrulline. The differences between the fast, and slow rates of carbamylation of the two types of lysyl residues in apoenzyme are between 20 and 50 times if we assume (see below) that the fast rate corresponds to carbamylation of the active site lysine, Lys-258, and the slow phase to random carbamylation of the other 18 lysyl residues in each subunit. The fast rate of cyanate incorporation, after correction for the slow contribution, is \( k = 0.12 \text{ min}^{-1} \) which is close to the first order rate constant of inactivation, \( k = 0.09 \text{ min}^{-1} \), obtained under identical experimental conditions.

Kinetics of pH Dependence of Enzyme Inactivation by Cyanate—The inactivation of aspartate transaminase by cyanate follows a pseudo-first order behavior at the pH tested between pH 7.59 and 8.91 (Fig. 5). A plot of these rate constants versus pH is sigmoidal with an inflexion point at pH 8.0. Because of the experiments described below we believe...
that the inactivation is due to carbamylation of an amine, from Lys-258. From the kinetics of inactivation using Equation 1 we (see “Experimental Procedure”) can evaluate the pH-independent second order rate constant of carbamylation and the pK of the amine. From the pseudo-first order rate constants ($k_{obs}$) we plot $C_t/k_{obs}$ versus $k_c/[H^+]$ (Fig. 5). This kinetic technique gives a pH-independent second order rate constant of $146 \pm 5 \, M^{-1} s^{-1}$ for the carbamylolation of an amine with a pK of $7.98 \pm 0.08$.

**Identification of $[^{14}C]$Cyanate-labeled Peptide**—The identification of the amino acid residues modified by cyanate was accomplished by isolation and characterization of the $[^{14}C]$-labeled peptide. Prior to tryptic hydrolysis both apoenzyme and apoenzyme$^*$ were carboxymethylated as indicated under “Experimental Procedure.” Passage of the tryptic peptides through a Sephadex G-50 column produced the elution profile in Fig. 6. The fractions containing the main radioactivity peak were pooled to be purified in the DEAE-cellulose column, which gave the elution profile in Fig. 7. The radioactive material appears mainly at the beginning of the first gradient and in a later fraction at high salt concentration. In the apoenzyme most of the radioactivity belongs to the first fraction. On the other hand, previous treatment of the holoenzyme with nonradioactive KNCO produces apoenzyme, which after treatment with $[^{14}C]$cyanate shows an elution pattern at the bottom of Fig. 7, with a marked decrease of radioactivity in the first peak. The differences in radioactivity of the first peak are also consistent with the total diminution of radioactivity incorporated (Table I and Fig. 3) by apoenzyme$^*$. The fractions between tubes 80 and 90 contain peptides with identical amounts of radioactivity in apoenzyme or apoenzyme$^*$. Amino acid composition of this fraction did not match any tryptic peptide in the sequence of aspartate transaminase (20, 21). Further purification in a Sephadex G-10 column and paper chromatography shows a single radioactive fluorescamine and/or ninhydrin-positive spot which upon acid hydrol-
ysis had the composition (Table II) of the tryptic peptide containing Lys-258, which in native transaminase has the following sequence: Try-Phe-Val-Ser-Glu-Gly-Phe-Glu-Leu-Phe-Cys-Ala-Gln-Ser-Phe-Ser-Phe-Ser-Lys-Asn-Phe-Gly-Leu-Try-Asn-Glu-Arg.

Tryptic hydrolysates of KN\textsuperscript{14}CO-treated holoenzyme produce a DEAE-cellulose elution profile where Fractions 80 to 90 are void of radioactivity and where the large early radioactivity peak is present.

Co-enzyme Binding to Cyanate-labeled Apoenzyme—Apoenzyme\textsuperscript{*} or native apoenzyme after carbamylation of Lys-258 can bind pyridoxal-P. Binding of the co-enzyme can be determined by fluorescence quenching (15, 22). By this method we detect binding of one pyridoxal-P per subunit of transaminase. Conversion of Lys-258 to homocitrulline blocks the amine which is thus unable to form a Schiff base with the aldehyde of pyridoxal-P, hence, the unusual spectrum with a maximum of 390 nm (Fig. 8). The union with the co-enzyme is strong and separation cannot be achieved by dialysis or passage through a Sephadex G-25 column. Conversion of the amine to the ureido group does not appear to create much steric hindrance at the active site. The modified enzyme binds co-enzyme, and addition of an amino acid substrate produces the immediate formation of a complex with the characteristic absorption maximum (430 nm) of enzyme:substrate complexes (23) where the substrate amino acid forms an internal Schiff base with the co-enzyme. The complex does not appear to be of an abortive type since it is able to convert, albeit very slowly, to a pyridoxamine complex spectrum with absorbance at 330 nm. Monitoring the properties of the latter mixture shows production of keto acid..\textsuperscript{3}

### Amino acid composition of radioactive peptide

The radioactive peptide was isolated from the tryptic digest of a carboxymethylated and carbamylated holoenzyme followed by treatment of the apoenzyme with KN\textsuperscript{14}CO by the procedure described under "Experimental Procedure."

![Fig. 8. Spectral changes of carbamylated supernatant aspartate transaminase apoenzyme upon the addition of pyridoxal-P and amino acid to a solution (3.5 mg/ml) of carbamylated apoenzyme in 0.1 M Tris-Cl buffer, pH 8.0. 1, Carbamylated apoenzyme and pyridoxal-P; 2, same as 1 immediately after addition of 20 mM sodium glutamate; 3, same as 2 after 8 h; 4, same as 2 after 24 h.](attachment:fig8.png)

**Table II**

| Amino acid       | Residues | Experimental | Theoretical |
|------------------|----------|--------------|-------------|
| Lysine           | 9.20     | 0.41         |             |
| Homocitrulline   | 12.57    | 0.56         |             |
| Arginine         | 17.73    | 0.79         | (1)         |
| Carboxymethylcysteine | 20.35   | 0.91         | (1)         |
| Aspartic acid    | 44.87    | 2.00         | (2)         |
| Serine           | 69.11    | 3.08         | (3)         |
| Glutamic acid    | 86.39    | 3.85         | (4)         |
| Glycine          | 50.49    | 2.25         | (2)         |
| Alanine          | 25.33    | 1.04         | (1)         |
| Valine           | 22.44    | 1.00         | (1)         |
| Leucine          | 38.80    | 1.72         | (2)         |
| Tyrosine         | 33.88    | 1.51         | (2)         |
| Phenylalanine    | 97.17    | 4.33         | (5)         |

\*Represent the numbers of residues relative to valine.

DISCUSSION

Cyanate treatment of proteins is most likely to result in stable derivatives of amino groups such as the $\epsilon$-amino group of lysine or the terminal $\alpha$-NH$_2$ group (19, 24). The products of cyanate reaction with other amino acid residues decompose in aqueous solutions at neutral pH (19, 24).

In supernatant aspartate transaminase KNCO reacts with both NH$_2$-terminal and lysyl residues with formation of carbamylated alanine (NH$_2$-terminal residue) and homocitrulline. Selection of the groups modified is based on the dissimilarity of pK values of different amino groups, since carbamylation requires the nonprotonated amine (19, 24). Susceptibility to carbamylation is further enhanced since at least one lysine is protected by the pyridoxal P chromophore. Thus, carbamylation of holoenzyme should lead, as observed, to modification of the NH$_2$-terminal alanine and some of the nonactive site lysyl groups. The active site Lys-258 becomes exposed only in the apoenzyme and if its pK is abnormal preferential carbamylation of Lys-258 should occur.

The pH dependence of inactivation of the apoenzyme\textsuperscript{*} is consistent with carbamylation at amino groups where isocyanate reacts with an unprotonated amine with a pK value of 7.98 ± 0.08 (Fig. 5). The data are most consistent with assigning this pK to the $\epsilon$-amino group of Lys-258. The pK value as determined by the chemical modification method is similar to that value detected for a group at the active site using an NMR spectroscopic procedure after the insertion of a $^{19}$F marked probe (15). The pH independent second order rate constant, $k = 148 ± 5 \text{ M}^{-1}\text{s}^{-1}$, calculated from the data in Fig. 5 is within the range that can be expected for cyanate reactivity with amines of similar pK (7, 8). Indeed it is very close to those rate values measured during carbamylation of the NH$_2$-terminal of the $\alpha$ chains of deoxymyoglobin, with pK 7.7 ± 0.3 (8).

Because after carbamylation of apoenzyme\textsuperscript{*} with concomitant loss of catalytic activity the only new radioactive tryptic peptide appearing contains Lys-258 we believe that inactivation is due to the specific carbamylation of the active site residue. Acid hydrolysis of the Lys-258-containing peptide does not give a complete recovery of lysine as homocitrulline because of conversion of homocitrulline back to lysine in approximately 30% yield, within the range observed in acid hydrolysis of homocitrulline in proteins (7, 19, 25), and is identical with the yield of our acid hydrolyzed homocitrulline internal standard.

The blocking of the NH$_2$-terminal groups with full retention of catalytic activity is to our knowledge the first such observation in aspartate transaminase and connotes the lack of participation of the NH$_2$-terminal alanine in the active site or any essential function in this enzyme. It may also indicate a distal location of the NH$_2$-terminal group with respect to the chromophore since subsequent to carbamylation of Lys-258 no

\*J. C. Slebe and M. Martinez-Carrion, manuscript in preparation.
steric hindrance or spectrophotometric anomaly in the active site chromophore is observed.

The method of using the pH dependence of the rate of carbamylation appears to be a useful procedure in detecting unusual lysyl residues with pK values considerably lower than those in the rest of the protein (7, 8).

Since the kinetics of inactivation give no evidence of deviation from pseudo-first order kinetics, the possibility of hindrances or of any cooperativity effects by the other residues reacting with cyanate is unlikely. Conformational or other structural changes induced by the modification of the non-active site lysine residue or the NH₂-terminal group are ruled out by the retention of catalytic activity in carbamylated holoenzyme. These observations are consistent with other results in which acetylation or succinylation of almost 80% of all amino groups in aspartate transaminase does not significantly alter catalytic activity (26–28). In the apoenzyme the effect of some carbamylation of amino groups extraneous to the active site is more difficult to assess. However, since after cyanate treatment pyridoxal-P binds stoichiometrically and the resulting holoenzymes appear to undergo a half transamination upon addition of amino acid, a large conformational change is unlikely.

All enzymes in which pyridoxal-P participates in catalysis bind the co-enzyme as the Schiff base with the ε-amino group of a lysyl residue. Among these enzymes the transaminases not only bind pyridoxal-P but also pyridoxamine-P. From these facts and the present observations it can be concluded that the active site lysine is not a major contributor to the equilibrium constant in transaminases. The lysyl residue must also occupy a spot with a considerable degree of freedom in the active site topography, where not even the introduction of the carbamyl moiety significantly perturbs pyridoxal-P. After carbamylation of Lys-258 the co-enzyme seems to form the internal aldime with an amino acid substrate with full retention of initial steric discrimination, as D amino acids are not accepted. The initial enzyme-co-enzyme-substrate complex of holoenzyme carbamylated at Lys-258 must be held in a new form of the enzyme (active site carbamylated) with a con-enzyme attached at the active site and able to accept substrate may yet provide the best tool for the study of the functional role of Lys-258.

Finally, we should note that this new awareness of low pK ε-amino groups in pyridoxal-P proteins should be considered as a possible trouble spot in cyanate treatment of sickle cell patients (29). The susceptibility of the unusual lysyl residues to carbamylation is of the same order of magnitude as reported for the NH₂-terminal group of human hemoglobin. The necessary long term cyanate therapy in sickle cell patients is bound to perturb the pyridoxal-P enzymes, particularly those enzymes with low affinity for pyridoxal-P or in individuals with low pyridoxal diets or pathologic conditions where a protective effect of the co-enzyme is likely to be minimal. In view of the reports that some neurological effects are observed in trial patients (30) subjected to cyanate treatment and a significant fraction of administered cyanate appears in the organs of experimental animals (29), and in view of the fact that pyridoxal-P plays an essential role in the metabolism of biogenic amines, the data reported in this paper should be explored further. Administration of large doses of Vitamin B₆ to cyanate-treated sickle cell patients appears to be a worthwhile melliorative procedure.

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