Mechanism of inactivation and identification of sites of modification of ornithine aminotransferase by 4-aminohex-5-ynoate

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Mechanism of Inactivation and Identification of Sites of Modification of Ornithine Aminotransferase by 4-Aminohex-5-ynoate†

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ABSTRACT: The inactivation of ornithine aminotransferase by an enzyme-activated irreversible inhibitor 4-aminohex-5-ynoate was accompanied by stoichiometric binding of the radiolabeled compound. Distribution of radiolabel among separated tryptic peptides indicated that more than one amino acid residue had reacted. Lys-292 and Cys-388 were positively identified. Reduction with borohydride was necessary to stabilize the adduct formed with Lys-292, and the relevant peptide prepared after this treatment contained equimolar amounts of inhibitor and coenzyme. The coenzyme chromophore in this peptide showed strong negative circular dichroism. A mechanism consistent with these observations is proposed.

The compound 4-aminohex-5-ynoate was designed and synthesized (Jung & Metcalf, 1975) as an enzyme-activated irreversible inhibitor with the intention of selectively inactivating 4-aminobutyrate aminotransferase because of the known anticonvulsant effects of raising the concentration of the inhibitory neurotransmitter 4-aminobutyrate in brain (Fowler & John, 1972; Anlezark et al., 1976). However, besides inactivating its intended target, it also inactivates several other pyridoxal phosphate dependent enzymes, notably, ornithine aminotransferase, glutamate decarboxylase, and, much more slowly, aspartate aminotransferase (Jung & Seiler, 1978; John et al., 1979). Of these enzymes, ornithine aminotransferase is perhaps the most appropriate to use in analysis of the

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chemistry of the interaction of aminohexynoate with pyridoxal-dependent enzymes. It is easily prepared in reasonably large quantities, and its full amino acid sequence has been determined (Mueckler & Pitot, 1985; Simmaco et al., 1986). Furthermore, it is known to be irreversibly and stoichiometrically labeled when [^{14}C]aminohexynoate is used to inactivate the enzyme (Jones et al., 1983). However, neither the site of binding to the enzyme nor the stability of the adduct after denaturation of the protein is known. In several cases in which a pyridoxal phosphate dependent enzyme has been inactivated by this type of inhibitor, the amino acid residue involved in covalent binding of the inhibitor is the lysine that normally forms an aldimine with the coenzyme [e.g., Morino and Okamoto (1973), Likos et al. (1982), Badet et al. (1984), and De Biase et al. (1989)], but in at least one case, that of bacterial pyridoxal-dependent histidine decarboxylase, a serine residue is labeled (Hayashi et al., 1986).

The intention of the experiments described in the present paper was to determine the identity of the amino acid residue labeled by the inhibitor and to produce evidence that might allow a choice to be made among possible inactivation mechanisms.

Materials and Methods

Materials. The inhibitor [2,3-^{14}C]-4-aminohex-5-ynoate (5.2 Ci mol^{-1}) was bought from Amersham International. Ornithine aminotransferase was prepared according to a modification (Williams et al., 1982) of the method of Peraino et al. (1969). TPCK[1]-treated trypsin was purchased from Sigma. Other reagents were from BDH Chemicals, Poole, Dorset, U.K., or from Carlo Erba, Milan, Italy.

Inactivation and Radiolabeling of Enzyme. Ornithine aminotransferase in 50 mM Hepps at pH 8.0 and containing 10 mM 2-oxoglutarate was treated with aminohexynoate at concentrations that varied according to the nature of the experiment. The reaction was monitored by observing the fall in absorbance at 420 nm that accompanies inactivation (John et al., 1979).

Denaturation, Proteolysis, and Purification of Peptides. Denaturation was achieved by adding guanidine hydrochloride to a final concentration of 4 M in 0.55 M Tris-HCl, pH 8.5, containing 2.3 mM EDTA and 6 mM dithiothreitol. The denaturation was conducted under nitrogen at room temperature in the dark for 3 h, and neutralized iodoacetate solution was added to a concentration of 20 mM. After 30 min the solution was dialyzed extensively. Digestion was achieved by adding trypsin in an amount 2% by weight of that of the denatured protein suspended in 0.1 M ammonium bicarbonate. After 1 h at 37 °C a second equal portion of trypsin was added and the digestion was continued for a further hour.

When complete analysis of all of the tryptic peptides was required, the digest was lyophilized and dissolved in 0.1% TFA. Peptides were then separated both analytically and preparatively by HPLC using a C-3 reversed-phase column (Beckman Ultrapore, 5 μm, 4.6 × 75 mm). In the preparative separations, a Beckman Model 332 HPLC apparatus equipped with a model 165 dual-wavelength detector was used. The absorbance of the eluate was monitored both at 220 nm and at 325 nm. The separation was developed by use of the gradient described in Figure 2. When necessary, repurifications were performed by using a C-8 reversed-phase column (Aquapore RP-300, 4.6 × 250 mm) and the same solvent system. Alternatively, a system in which solvent B was replaced by 4:1 acetonitrile/2-propanol was used. Analytical separations were also carried out with the solvent system described in Figure 2 but using a Milton Roy CM 4000 multiple solvent delivery system equipped with a Spectro Monitor 3100 variable-wavelength detector and a Beckman 171 radioisotope detector. When preparations of the single large peptide derived from reduced enzyme samples was required, a column (20 × 1 cm) of G-25 fine Sephadex was used in 0.1 M ammonium bicarbonate.

Analyses of Amino Acid Content and Sequence. Acid hydrolysis was performed in 6 M HCl for 24 h at 110 °C in vacuo, and the amino acids were separated and quantified on an LKB 4151 Alphaplus amino acid analyzer. Sequence analysis was performed with an Applied Biosystems Model 470A gas-phase protein sequencer equipped with a Model 120A PTH analyzer for the on-line determination of PTH amino acid derivatives. Numbering of amino acid residues is based on the cDNA-derived sequence (Mueckler & Pitot, 1985).

Determination of Radioactivity. Radioactivity was determined by use of a Beckman Model LS 6800 liquid scintillation counter and Biofluor liquid scintillation cocktail purchased from New England Nuclear.

Absorption Spectra and Circular Dichroism. Absorption spectra were determined either with a Beckman Model 25 or with a Beckman Model DU7 spectrophotometer. CD of samples was determined by using a Dichrographe III, CNRS, Roussel, Jouan.

Results and Discussion

Stoichiometry and Stability of Labeling. Products of the enzyme inactivation process are likely to contain imines, the reduction of which by sodium borohydride would be expected to stabilize the linkage between coenzyme, protein, and inhibitor. For this reason the inactivated enzyme (5 mg, 108 nmol) was divided into two equal 1-mL portions, one of which was treated with sodium borohydride (approximately 10 μg). Both samples were separated from unbound inhibitor by repeated cycles of forced dialysis and redilution using Centricon-30 microconcentrators (Amicon) until, after six cycles, the radioactivity of the separated low molecular weight fraction was zero. At this stage the radioactivity due to protein-bound inhibitor corresponded to 0.89 and 0.74 mol/mol of active site for the reduced and unreduced enzyme samples, respectively. Bearing in mind the fact that preparations of ornithine aminotransferase invariably contain approximately 20% of enzyme with coenzyme inactively bound (Sanada et al., 1976), these results confirm earlier observations (Jones et al., 1983) that the reaction is very close to being stoichiometric and that it is irreversible over this time scale, at least when the enzyme remains folded. Each sample of enzyme (54 nmol) was denatured and carboxymethylated before being dialyzed for 2 h against 10 mL of water. Determination of radioactivity in the solutions passing through the dialysis tube at this stage showed that material equivalent to 3.6 and 5.1 nmol of inhibitor was released from the reduced and unreduced samples, respectively. These dialysates were dried by lyophilization and dissolved in a final volume of 0.7 mL of water, and absorbance spectra were determined. The unreduced enzyme released material that had an absorbance maximum near 420 nm whereas the reduced enzyme released coenzyme absorbing at about 330 nm.

Distribution of Radiolabel. Both reduced and unreduced samples of radiolabeled enzyme were digested with trypsin.

[1] Abbreviations: TPCK, tosylphenylalanine chloromethyl ketone; TFA, trifluoroacetic acid; CD, circular dichroism; PTH, phenylthiohydantoin.

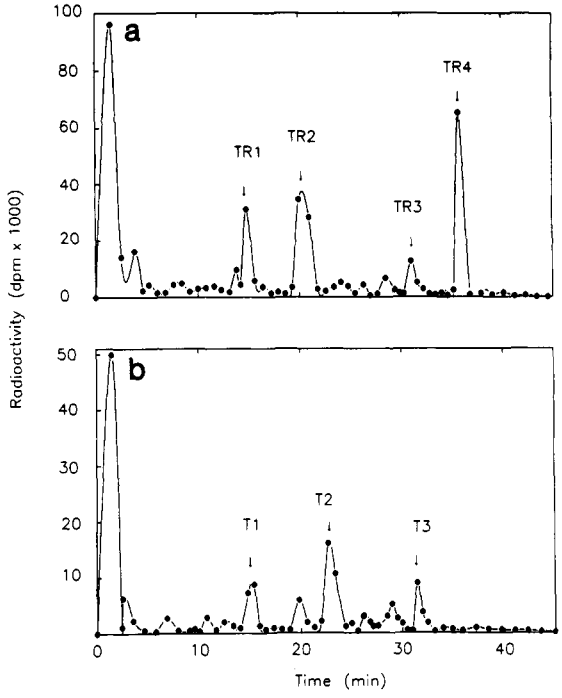


FIGURE 1: Distribution of radioactivity in preparative separations of tryptic digests. Tryptic digests of denatured ornithine aminotransferase, inactivated by use of 1.3 mM [^{14}C]aminohexynoate, were separated by reversed-phase HPLC as described in the text and using the gradient described in Figure 3. Column eluate was monitored at 220 and 325 nm. The pattern of radioactivity in the case of the sample from the reduced enzyme (a) was obtained from chromatography of all 2.5 mg of the sample whereas in the case of the digest from the unreduced sample (b) the radioactivity pattern is from a portion (0.8 mg).

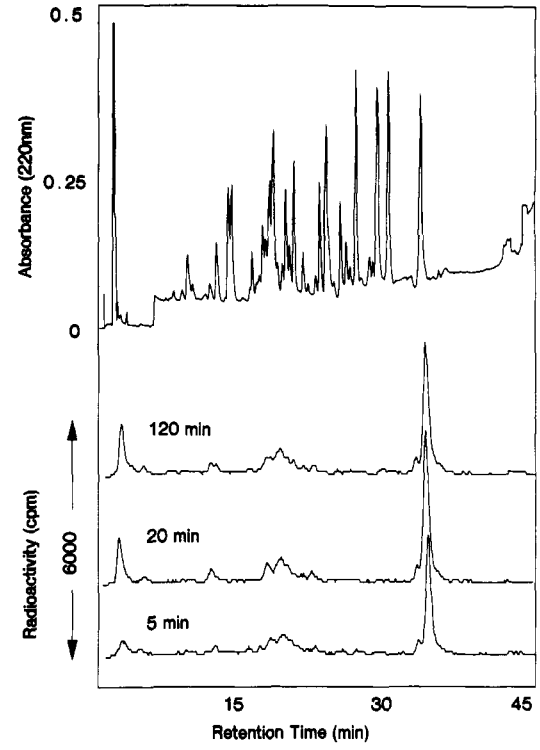


FIGURE 2: Analysis of tryptic digests of ornithine aminotransferase after rapid inactivation by aminohexynoate and delayed reduction. Inactivations were conducted by use of 10 mM [^{14}C]aminohexynoate. Sodium borohydride (1 mg/mL) was added at the intervals indicated. Denaturation and tryptic digestion were as described in the text. Chromatographic conditions: flow rate, 1.5 mL/min; solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in 70% acetonitrile in water. For elution, after 2 min at 0% solvent B, a gradient from 0% to 50% solvent B was developed in 30 min, followed by 6 min at 50% and a gradient over 2 min to 95% solvent B; the column was then washed for 5 min at 95% solvent B.

The spectra of the tryptic digests, although having absorbance in the region of 330 nm, did not show a clear maximum. Assay of radioactivity showed that 46 and 38 nmol of inhibitor-derived material was present in the digests produced from reduced and unreduced samples, indicating that reduction with borohydride is not required for retention of the majority of the inhibitor even by the denatured protein. Digests were fractionated by HPLC. Fractions were collected peak by peak, and the radioactivity associated with each fraction was determined (Figure 1). The preparative nature of this experiment required a high loading of the column, so that many A_{220} peaks were off scale, and they are therefore not shown. Clearly, radiolabel is distributed among many fractions of the chromatogram. The digest from the unreduced sample (Figure 1b) differed in that the major radiolabeled and 325 nm absorbing peak (TR4, Figure 1a) at the end of the chromatogram was completely absent. Furthermore, no peak detectable by absorbance at 220 nm was present in this position. One other significant difference between the chromatograms from reduced and unreduced samples of the enzyme was that the amount of radioactivity eluting near the front was approximately 50% larger, proportionally, in the case of the unreduced sample.

The fact that without borohydride reduction the radiolabel associated with TR4 was lost raised the possibility that the labeling pattern arose from transfer of an inhibitor derivative from its initial site to other residues that bind it more stably. In order to test this possibility, we conducted an experiment in which the enzyme was inactivated with a concentration of [^{14}C]aminohexynoate (10 mM) high enough to inactivate 80% of the enzyme in 5 min. Samples were reduced with borohydride at this time and at intervals up to 2 h. The results of these analytical HPLC analyses (Figure 2) are very similar to each other, suggesting that no migration of label occurs.

Analysis of Peptides. The radioactivity associated with the major peak (TR4) that eluted toward the end of the chromatogram obtained from the reduced sample (Figure 1a) indicated the presence of 6.0 nmol of bound inhibitor. This material was subjected to sequence analysis, and the amount of radioactivity associated with each cycle of the Edman degradation was determined. A clear single sequence was obtained and found to correspond to a portion of the sequence beginning at Trp-275. The absorbance spectrum of the peptide showed a definite 320 nm absorbing chromophore which, to conform to the amount of radiolabel present, requires an extinction coefficient of 7200 L mol^{-1} cm^{-1}, a value that agrees well with those of other pyridoxyl compounds (Kallen et al., 1985). The amount of peptide material present in the sample was determined by subjecting a portion of it to complete acid hydrolysis followed by quantitaive analysis of the separated amino acids. This analysis gave a figure of 6.5 nmol of peptide material. Thus, 1 mol of this peptide is bound to 1 mol of coenzyme and 1 mol of inhibitor.

Identification of the radiolabeled residue in TR4 was not straightforward. The peptide contains Lys-292, which is the residue to which the coenzyme binds and which is labeled in analogous inhibitions of other aminotransferases. At the cycle (cycle 18) corresponding to Lys-292 no PTH derivative was detectable despite the fact that the alanine, leucine, and serine expected from the next three cycles were very clearly present. Some radioactivity was associated with the 18th cycle, but the first major extraction of radioactivity coincided with the 19th

cycle, where alanine was both expected and found. The extracts from the next two cycles contained similar amounts of radioactivity, with the maximum at cycle 20 (leucine). The unreactive nature of the side chains of alanine and leucine, together with the fact that their PTH derivatives were detected and chromatographed normally, makes it very unlikely that they have reacted with the inhibitor. The abolition of susceptibility to tryptic digestion shows that lysine-292 is modified, and circular dichroism of the peptide (presented below) shows that the modification is not simply to pyridoxyllysine. We propose that, as in several other examples of similar inactivation reactions in which the corresponding lysine does react (Morino & Okamoto, 1973; Likos et al., 1982; Badet et al., 1984; De Biase et al., 1989), Lys-292 has reacted to produce a derivative containing both inhibitor and coenzyme. We propose additionally that the chemistry of the unusual PTH-amino acid derivative means that it does not extract normally into the solvents at the appropriate cycle of Edman degradation, but instead it is extracted as a carry-over into subsequent steps of the analysis. The fact that the large peptide corresponding to TR4 was not present in the tryptic digest of the unreduced sample indicates that without reduction the bond formed with the side chain of Lys-292 is labile.

The peaks (TR1 and T1, Figure 1) accounted for approximately 8% of the eluted radioactivity from both reduced and unreduced samples. This material required further purification (see Materials and Methods), and, from both samples, pure peptides were obtained having sequences corresponding to a portion of the protein beginning at Asp-387 and continuing for six residues to Lys-392. Radioactivity, stoichiometric with the peptide itself, was released at the second cycle of the Edman degradation, indicating clearly that Cys-388 was labeled. However, no PTH-cysteine was observed; instead, a PTH-amino acid was found eluting near the middle of the chromatogram in a position close to that of dehydroserine. The amount of radioactivity released at this cycle indicated stoichiometry between this residue and inhibitor. Because only 8% of the total bound radiolabel was associated with this peptide, we conclude that modified and unmodified forms of this peptide have been separated. Absorbance analysis indicated that no pyridoxal derivative was present on the peptide.

The absorbance (220-nm) trace in the chromatogram from the unreduced sample showed a peak (T3, Figure 1b) associated with radioactivity corresponding to 5% of that applied but without significant absorbance at 325 nm. The clear sequence obtained indicated that this peptide began at Ala-293, the residue immediately after the active site lysine. This peptide was obtained in high yield, and sequence data were particularly good. No trace of peptide TR4 was present. In this case the peptide contained only 0.04 mol of radioactivity/mol of peptide, and it was not possible to assign it to any cycle of the sequence analysis. The low level of radiolabel demonstrates that the sample is a mixture containing predominantly the unlabeled peptide. The high yield of this peptide confirms that in the unreduced sample most of the Lys-292–Ala-293 bonds were subject to tryptic hydrolysis and therefore almost certainly contained unmodified lysine despite the observation that each mole of enzyme still contained 0.75 mol of radiolabel that did not separate by dialysis even from the denatured enzyme. Thus we conclude that, if the enzyme is not reduced, the adduct formed between lysine, coenzyme, and inhibitor eventually dissociates completely but, under the conditions of the inactivation, the protein still contains substantial amounts of tightly bound inhibitor which probably elutes in the large peak of radioactivity at the front of the chromatogram of the tryptic digest.

The profile of radioactivity between 16 and 29 min shows the presence of several poorly separated radiolabeled peptides. The material eluting in this region from the preparative separations (TR2) contained a total of approximately 14% of the radioactivity applied to the column. These radioactive fractions were combined and subjected to further purification. Sequence analysis of the resulting separated material still showed the presence of more than one peptide in each case. Although it was not possible to determine which residues were labeled, it was clear that no part of the sequence of peptides TR4 or TR1/T1 was present, indicating that the inhibitor must have reacted with residues other than Lys-292 or Cys-388. The presence of this radiolabeled material in similar amounts in the chromatogram from both reduced and unreduced samples showed that, as with Cys-388, the bonds formed with the inhibitor do not require reduction for stability.

Chirality of Products. Two different mechanisms have been proposed for the final stages of this type of inactivation (Jung & Metcalf, 1975; Ueno et al., 1982), and these result in differences in substitution at 4′-C of the coenzyme. To distinguish between these mechanisms, CD spectra of the inactivated reduced enzyme after unfolding in guanidine hydrochloride and of a purified sample of the peptide TR4 derived from it were determined.

Figure 3a shows CD and absorbance spectra of the native and borohydride-reduced enzyme samples. Both the 415 nm absorbing internal aldimine and the product of its reduction by sodium borohydride show strong positive CD which may be attributed to the asymmetric environment provided by the enzyme protein. The 320 nm absorbing chromophore arising from inactivation by aminohexynoate (Figure 3b) also shows positive CD, but the magnitude of the signal is significantly lower than that observed in the borohydride-reduced native enzyme. Treatment of the inactive enzyme with borohydride made no difference to its CD or absorbance spectra. Denaturation with guanidine resulted in complete loss of the positive CD signal at 320 nm and its replacement by a negative CD peak (data not shown). The large tryptic peptide (TR4) containing inhibitor and chromophore was purified and found to have a significant negative CD peak at 320 nm (Figure 4a). The possibility that the clear CD of these samples arose from residual structure in the large peptide was excluded by the results of another experiment in which the native enzyme was simply reduced to provide a sample of pyridoxyl-enzyme without asymmetry at 4′-C. The enzyme was denatured and digested in order to prepare the corresponding large pyridoxyl-peptide. Immediately after addition of guanidine, the strong positive CD signal at 325 nm disappeared completely (data not shown). The absence of CD in the purified pyridoxyl-peptide (Figure 4b) confirms the conclusion that it is not sufficiently structured to confer detectable asymmetry on the pyridoxyl chromophore. We therefore interpret these data by proposing that, before denaturation, the observed positive CD signal of the inactive enzyme (Figure 3b) is the summation of a large positive contribution arising from the asymmetric environment of the intact protein and a negative contribution arising from a newly formed asymmetry near the chromophore itself.

The negative 320-nm CD signal of the peptide provides strong evidence for the presence of an asymmetry at 4′-C of the coenzyme in the inhibited enzyme.

Mechanism of Inactivation. Several features of the mechanism of inactivation of ornithine aminotransferase by aminohexynoate have already been clearly demonstrated. Jung

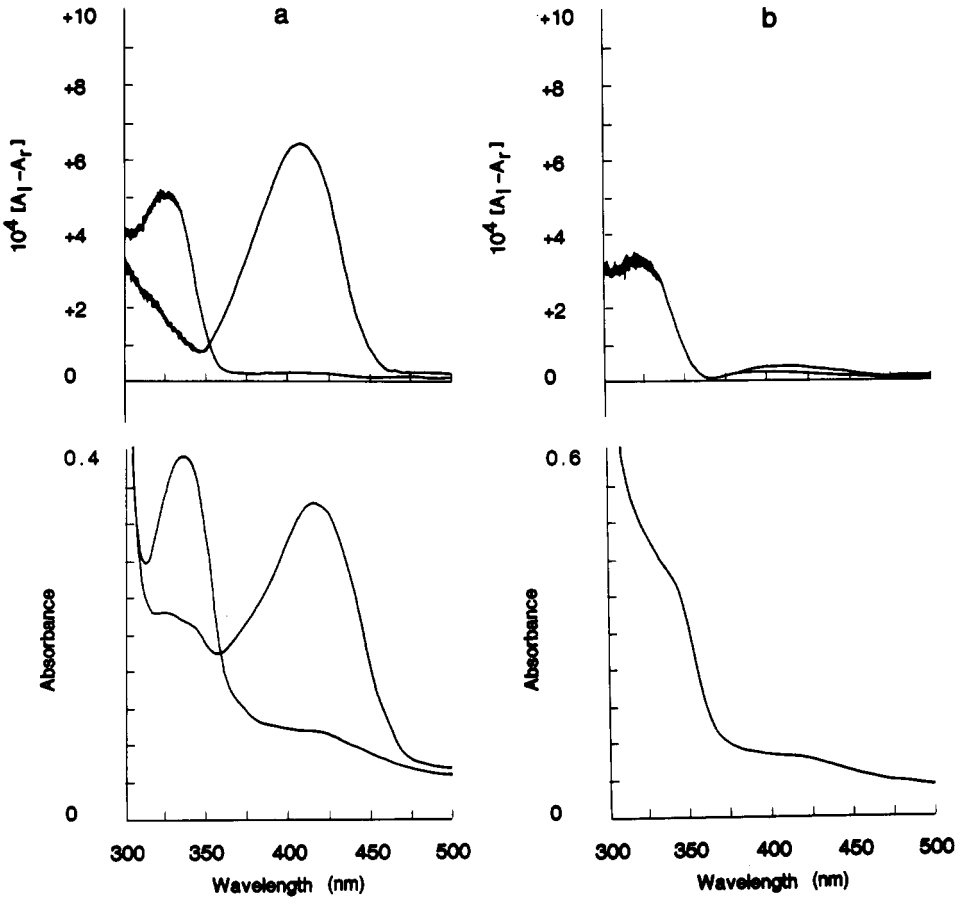


FIGURE 3: Absorbance and CD spectra of native and inactive ornithine aminotransferase. Ornithine aminotransferase (50 μM) was dissolved in 50 mM Hepps, pH 8.0, containing 10 mM 2-oxoglutarate. (a) Spectra of the native and borohydride-reduced enzyme without treatment with aminohexynoate. The spectra of the unreduced enzyme are those with 420-nm maxima. (b) Spectra of the aminohexynoate-inactivated enzyme before and after reduction.

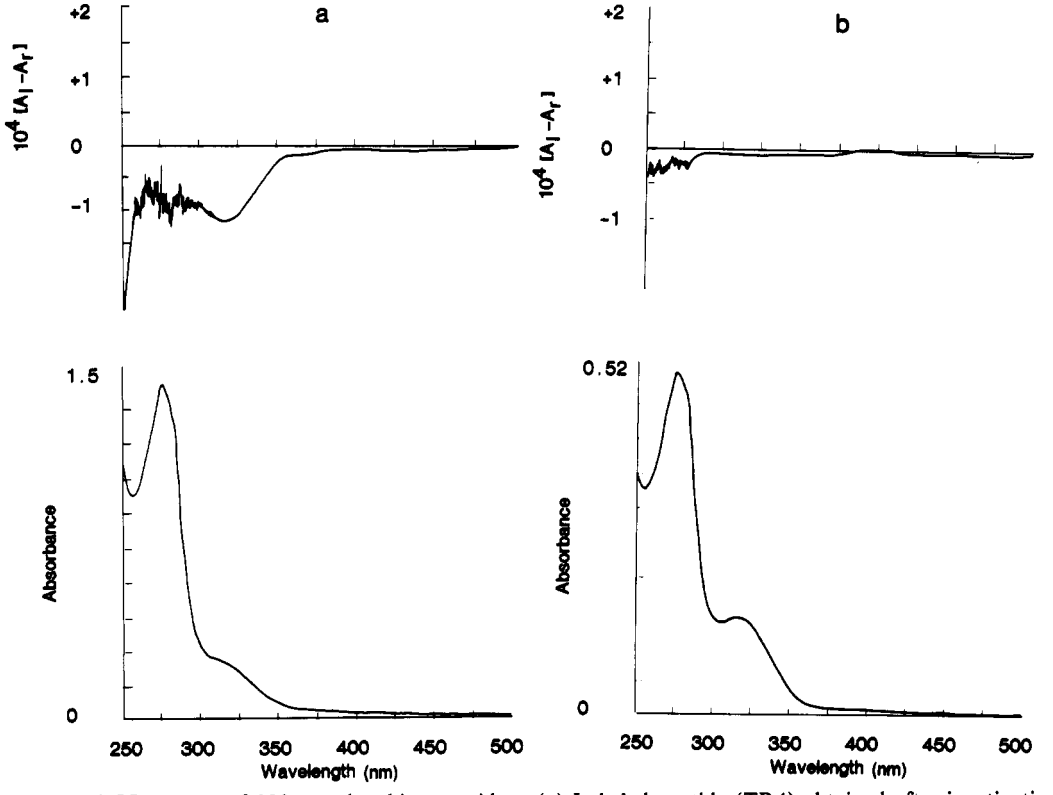
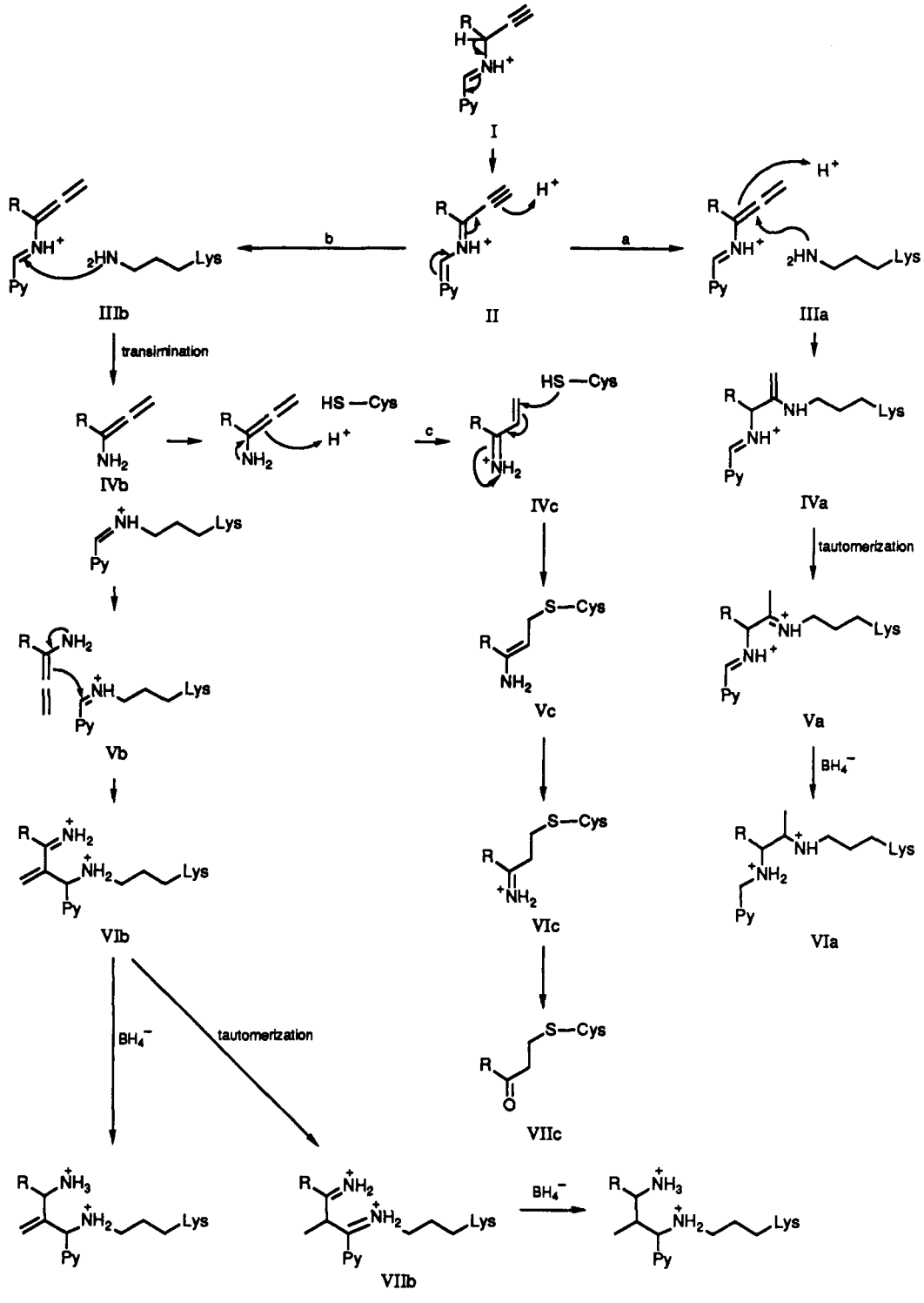


FIGURE 4: Absorbance and CD spectra of 320 nm absorbing peptides. (a) Labeled peptide (TR4) obtained after inactivation of ornithine aminotransferase (20 mg) with aminohexynoate and reduction with borohydride. (b) Pyridoxyl-peptide arising from borohydride reduction of ornithine aminotransferase (10 mg) that had not been treated with aminohexynoate.

Scheme I: Proposed Mechanisms for the Reaction of Ornithine Aminotransferase with Aminohexynoate



and Seiler (1978) showed that the specificity of the inactivation is for the enzyme in its pyridoxal form. Neither the apoenzyme nor the pyridoxamine form in the presence of ornithine was inactivated. This conclusion was reinforced by the observation (John et al., 1979) that, in the absence of added keto acid, inactivation proceeds only to 25% because aminohexynoate itself converts the enzyme to its pyridoxamine form in a simultaneous reaction which makes it insusceptible to further inactivation. Further evidence that the process is not only specific but requires enzyme activation comes from the observations that only the *S*-enantiomer of aminohexynoate inactivates the enzyme and that the 4-deuterated compound shows a primary isotope effect. The inability of the compound to radiolabel other proteins has been demonstrated by an experiment in vivo which showed that the only liver protein radiolabeled after intraperitoneal injection of [^{14}C]aminohexynoate chromatographed with ornithine aminotransfease (Jones et al., 1983).

In most cases [e.g., Morino and Okamoto (1973), Likos et al. (1982), Badet et al. (1984), and De Biase et al. (1989)] where enzyme-activated irreversible inhibitors have been used to inactivate pyridoxal phosphate dependent enzymes the radiolabel has been found to be bound to the same lysine that normally forms a Schiff base with the coenzyme.

Two fundamentally different mechanisms have been proposed for the final stages of inactivation by "suicide" inhibitors

of pyridoxal phosphate dependent enzymes. Both mechanisms share the same initial steps which in the case of aminohexynoate lead to the formation of the conjugated allene (III, Scheme I). Jung and Metcalf (1975) originally proposed that inactivation took place by the reaction of coenzyme-bound, conjugated allene with an enzyme nucleophile. Attack by the lysine amino group, as shown in route a, would lead initially to IVa, which can be expected to tautomerize to the diimine Va. Reduction with borohydride would then give the product VIa in which the coenzyme is linked to enzyme protein through the inhibitor derivative. This would leave the 4′-C of the coenzyme as CH_2 so that the nearest chiral center is separated from the coenzyme chromophore by three single bonds which would not account for the observed strong CD (Barrett, 1972).

An alternative and more complex mechanism of the type clearly shown to occur in the inactivation of aspartate aminotransferase and glutamate decarboxylase by serine *O*-sulfate and of GABA-aminotransferase by 4-amino-5-fluoropentanoic acid (Likos et al., 1982; Ueno et al., 1982; Silverman & Invergo, 1986) is indicated as path b, Scheme I. In this mechanism, addition of Lys-292 to 4′-C results in a "transimination" reaction which restores the original internal aldimine and liberates the aminoallene derivative of aminohexynoate (IVb). After rotation, Vb, the aminoallene, can react with the reformed internal aldimine to yield a compound, VIb, with asymmetry at 4′-C. We suggest that this is the initial product of the inactivation of ornithine aminotransferase by aminohexynoate. Tautomerization can again lead to a diimine, VIIb, which may in time be hydrolyzed if it has not been reduced. The adduct of inhibitor, coenzyme, and protein will be stabilized by borohydride reduction at any stage but will always retain the chiral center at 4′-C. Tryptic digestion would then give a peptide with the absorbance, circular dichroism, and radioactivity observed in TR4.

Reaction with the electrophilic center provided by 4′-C of the internal aldimine requires the nucleophilic reactivity expected of the aminoallene in IVb. Reaction with cysteine, or with any other amino acid side chain, requires a compound with electrophilic properties such as would be conferred on the aminoallene by its existence as an aldimine with pyridoxal phosphate as in IVa. However, absorbance analysis showed that none of the other radiolabeled peptides contained a derivative of pyridoxal phosphate, and we propose that path c is responsible for the labeling of Cys-388 and other unidentified residues. Path c entails protonation of the aminoallene to give the vinyl derivative 4-iminohex-5-enoate (IVc), a compound that would be expected to be highly reactive toward nucleophilic amino acid side chains as indicated. An alternative route that would modify cysteine or other nucleophiles has been proposed by Miles (1978) for the inactivation of tryptophan synthase by vinylglycine. In this mechanism, the aldimine with aminohexynoate would first be converted to the ketimine by partial transamination before nucleophilic addition took place at the terminal carbon of the alkyne group, eventually resulting in a compound analogous to a cyanine. This mechanism seems unlikely on the grounds that, after reduction, the resulting peptide would be expected to contain coenzyme but the absorption spectrum of the cysteine-containing peptide described in the present paper indicates that it does not. The expected stability of cyanines and analogous compounds suggests that release of coenzyme by rapid hydrolysis before addition of borohydride is unlikely.

A full explanation of these results must reconcile the stoichiometry of radiolabeling during the inactivation process with the multiple sites of binding of the radiolabel. A classical precedent for this behavior is the inactivation of ribonuclease by iodoacetate which carboxymethylates His-12 or His-119 but never both (Crestfield et al., 1963). In that case the explanation is that one of the active site histidines in its protonated state aligns the iodoacetate via a salt bridge with its carboxylate group so as to react with the other through its iodomethyl group. A mechanism of this type seems unlikely to be the explanation for the multiplicity of residues radiolabeled in the reaction of [^{14}C]aminohexynoate with ornithine aminotransferase.

The inactivation and radiolabeling of histidine decarboxylase by (fluoromethyl)histidine has interesting similarities with the system described in the present paper in that, if the inactivated decarboxylase is reduced very soon after inactivation, a single serine residue is radiolabeled (Hayashi et al., 1986). However, it was recently demonstrated (Bhattacharjee & Snell, 1990) that the primary product of the inactivation is the adduct corresponding to VIb of Scheme I and that the involvement of serine is by subsequent substitution into this adduct.

Random radiolabeling of the residues involved by a nonspecific mechanism can be ruled out on the grounds that in three experiments, conducted on separate occasions under slightly different conditions, the stoichiometry of radiolabeling is very close to 1 mol of inhibitor/mol of active site. It seems most likely that the stoichiometry of the system arises because all of the modifications require processing by the enzyme and also lead to its inactivation.

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Registry No. Lys, 56-87-1; Cys, 52-90-4; ornithine aminotransferase, 9030-42-6; 4-aminohex-5-ynoic acid, 57659-38-8; pyridoxal 5′-phosphate, 54-47-7.

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Interaction of Human Placental Ribonuclease with Placental Ribonuclease Inhibitor†

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ABSTRACT: The interactions of human placental ribonuclease inhibitor (PRI) with bovine pancreatic ribonuclease (RNase) A and human angiogenin, a plasma protein that induces blood vessel formation, have been characterized in detail in earlier studies. However, studies on the interaction of PRI with the RNase(s) indigenous to placenta have not been performed previously, nor have any placental RNases been identified. In the present work, the major human placental RNase (PR) was purified to homogeneity by a five-step procedure and was obtained in a yield of 110 μg/kg of tissue. The placental content of angiogenin was also examined and was found to be at least 10-fold lower than that of PR. On the basis of its amino acid composition, amino-terminal sequence, and catalytic properties, PR appears to be identical with an RNase previously isolated from eosinophils (eosinophil-derived neurotoxin), liver, and urine. The apparent second-order rate constant of association for the PR·PRI complex, measured by examining the competition between PR and angiogenin for PRI, is 1.9×10^8 M^{-1} s^{-1}. The rate constant for dissociation of the complex, determined by HPLC measurement of the rate of release of PR from its complex with PRI in the presence of a scavenger for free PRI, is 1.8×10^{-7} s^{-1}. Thus the K_i value for the PR·PRI complex is 9×10^{-16} M, similar to that obtained with angiogenin, and 40-fold lower than that measured with RNase A. Complex formation causes a small red shift in the protein fluorescence emission spectrum, with no significant change in overall intensity. The fluorescence quantum yield of PR and the Stern-Volmer constant for fluorescence quenching by acrylamide are both high, possibly due to the presence of an unusual posttranslationally modified tryptophan residue at position 7 in the primary sequence.

Despite several decades of study [see Levy and Karpetsky (1981)], human ribonucleases (RNases)[1] have only recently begun to be characterized as molecular entities. Five structurally and functionally distinct species have now been identified, including pancreatic RNase (Weickmann et al., 1981; Beintema et al., 1984), the "nonsecretory" RNase from liver, urine, and eosinophils (Cranston et al., 1980; Gleich et al., 1986; Beintema et al., 1988a; Sorrentino et al., 1988), and three additional forms found in blood (see Discussion). These RNases are closely related: their amino acid sequences are homologous and all cleave preferentially on the 3′ side of pyrimidines, albeit at different rates and with somewhat different specificities.

Contrasting with the considerable amount of knowledge obtained concerning the primary structures and, in some instances, functional properties of these enzymes is the relative scarcity of information available concerning their distribution throughout the body. Thus, blood, urine, pancreas, and liver remain the only sources from which human RNases have been purified to homogeneity and definitively identified. The nature of the RNases in other tissues has been explored thus far

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[1] Abbreviations: RNase(s), ribonuclease(s); RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; PR, placental ribonuclease; TSE, 20 mM Tris, pH 7.5, containing 0.25 M sucrose and 1 mM EDTA; CM, carboxymethyl; C18, octadecylsilane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mes, 2-morpholinoethanesulfonic acid; CpA, cytidylyl-3′,5′-adenosine; UpA, uridylyl-3′,5′-adenosine; HSA, human serum albumin; pHMB, *p*-(hydroxymercuri)benzoate; EDN, eosinophil-derived neurotoxin; PTH, phenylthiohydantoin; ECP, eosinophil cationic protein.

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