Resnick, R. M., & Nelsestuen, G. L. (1980) *Biochemistry 19*, 3028–3033.
Torbet, J., & Wilkins, M. H. F. (1976) *J. Theor. Biol. 62*, 447–458.
Van der Waart, P., Hemker, H. C., & Lindhout, T. (1984) *Biochemistry 23*, 2838–2842.
Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochemistry 17*, 1792–1801.
Wilkins, M. H. F., Blaurock, A. E., & Engleman, D. M. (1971) *Nature (London) New Biol. 230*, 72–76.
Witz, J. (1983) *Acta Crystallogr., Sect. A: Found. Crystallogr. A39*, 706–711.
Zaccai, G., & Jacrot, B. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 139–157.

# Rapid Degradation of D- and L-Succinimide-Containing Peptides by a Post-Proline Endopeptidase from Human Erythrocytes†

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*Received May 8, 1987; Revised Manuscript Received July 13, 1987*

ABSTRACT: We have been interested in the metabolic fate of proteins containing aspartyl succinimide (Asu) residues. These residues can be derived from the spontaneous rearrangement of Asp and Asn residues and from the spontaneous demethylation of enzymatically methylated L-isoAsp and D-Asp residues. Incubation of the synthetic hexapeptide *N*-Ac-Val-Tyr-Pro-Asu-Gly-Ala with the cytosolic fraction of human erythrocytes resulted in rapid cleavage of the prolyl–aspartyl succinimide bond producing the tripeptide *N*-Ac-Val-Tyr-Pro. The rate of this reaction is equal for both L- and D-Asu-containing peptides and is 10-fold greater than the rate of cleavage of a corresponding peptide containing a normal Pro–Asp linkage. When the aspartyl succinimide ring was replaced with an isoaspartyl residue, the cleavage rate was about 5 times that of the normal Pro-Asp peptide. The tripeptide-producing activity copurified on DEAE-cellulose chromatography with an activity that cleaves *N*-carbobenzoxy-Gly-Pro-4-methylcoumarin-7-amide, a post-proline endopeptidase substrate. These two activities were both inhibited by an antiserum to rat brain post-proline endopeptidase, and it appears that they are catalyzed by the same enzyme. This enzyme has a molecular weight of approximately 80 000 and is covalently labeled and inhibited by [$^3$H]diisopropyl fluorophosphate. The facile cleavage of the succinimide- and isoaspartyl-containing peptides by this post-proline endopeptidase suggests that it may play a role in the metabolism of peptides containing altered aspartyl residues.

The thermodynamic instability of proteins at physiological pH and temperature results in the spontaneous formation of a number of covalently altered derivatives of amino acid residues. Two of the least stable residues are aspartic acid and asparagine because they can be involved in deamidation, isomerization, and racemization reactions that result in the formation of D- and L-succinimidyl, D- and L-isoaspartyl, and D- and L-aspartyl residues (Ahern & Klibanov, 1985; Brunauer & Clarke, 1986; Di Donato et al., 1986; Geiger & Clarke, 1987). There is evidence that proteins and peptides containing at least two of these derivatives, L-isoaspartyl and D-aspartyl residues, can be methyl esterified by widely distributed protein methyltransferases (Clarke, 1985). In synthetic peptides these esters have been shown to be nonenzymatically demethylated to succinimide residues, which can then be hydrolyzed to give a mixture of D- and L-isoaspartyl and D- and L-aspartyl residues (Murray & Clarke, 1986; McFadden & Clarke, 1987). Although the cellular role of the protein methyltransferase is not understood, it has been proposed that this enzyme may function in the repair of altered proteins because it can catalyze the conversion of isoaspartyl residues in peptides to aspartyl peptides in moderate yield (Johnson et al., 1987; McFadden & Clarke, 1987).

In this study we have focused on an alternative pathway for the metabolism of proteins containing atypical aspartate derivatives. We were interested in examining the possibility that specific enzymes may catalyze the proteolysis of these species. We have used the synthetic peptide *N*-Ac-Val-Try-Pro-Asp-Gly-Ala as a prototype on which to build a series of abnormal peptides where the aspartyl residue is replaced with a D-succinimide, L-succinimide, D-isoaspartyl, or L-isoaspartyl derivative. Each peptide was then used as a model to explore the proteolytic metabolism of these altered protein structures in cell extracts. We have found that when these peptides are incubated with human erythrocyte cytosol, the Pro–X bond is rapidly cleaved by an activity that is immunologically related to a post-proline endopeptidase isolated from rat brain (Andrews et al., 1982). Post-proline endopeptidase has been described as a serine protease in several nonerythroid mammalian tissues that exists as a single polypeptide of approximately 70 000 daltons and an optimal activity observed between pH 7.5 and pH 8.0 (Rupnow et al., 1979; Walter et al., 1980). Interestingly, in our studies, the human erythrocyte enzyme was found to cleave the abnormal hexapeptides at a faster rate than the hexapeptide containing the normal Pro–Asp bond. The fact that the altered peptide is recognized as a better substrate than the normal substrate indicates that proteases may prefer damaged proteins over normal proteins and se-

† This work was supported by National Institutes of Health Grants GM-26020 and EY-04912 and by a Grant-in-Aid from the American Heart Association with funds contributed in part by the Greater Los Angeles Affiliate.
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0006-2960/87/0426-7798$01.50/0 

lectively rid the cell of nonfunctional proteins in this manner.

## Materials and Methods

*Peptides.* All peptides contain amino acids in the L configuration unless otherwise specified. The imide hexapeptide Val-Tyr-Pro-Asu-Gly-Ala and *N*-CBZ-Gly-Pro-4-methylcoumarin-7-amide (*N*-CBZ-Gly-Pro-MEC) were purchased from Vega Biochemicals (Tucson, AZ).[1] *N*-CBZ-Gly-Pro-Leu-Gly was from Peninsula Laboratories (Belmont, Ca). The D-imide hexapeptide Val-Tyr-Pro-D-Asu-Gly-Ala was manually synthesized with standard solid-phase procedures as previously described (Murray & Clarke, 1984). Tetragastrin imide was prepared as described (McFadden & Clarke, 1987).

*High-Performance Liquid Chromatography (HPLC).* The chromatography system was from Waters (two M-45 or two Model 510 pumps, M660 gradient programmer, U6K injector, Model 441 detector with Zn lamp, 214-nm filter, and 1-cm path length). Alltech reverse-phase columns were used for all peptide separations (4.6 × 250 mm Econosphere C18; 5-μm spherical particles). Solvent A was 0.1% (w/v) trifluoroacetic acid and solvent B was 0.1% (w/v) trifluoroacetic acid in 9:1 (v/v) acetonitrile–water. The separations were performed at room temperature. Chromatograms were recorded and peak areas integrated (base line to base line) by a Shimadzu C-R3A integrator.

Peptides were purified by dissolution in solvent A and injection onto the HPLC system described above. Elution was by an increasing linear gradient of 0.5% solvent B per minute at a flow rate of 1 mL $min^{-1}$. The peptides were collected, lyophilized, and stored over desiccant in the dark at −20 °C.

*Preparation and Characterization of N-Acetyl-Val-Tyr-Pro-Asu-Gly-Ala.* The imide hexapeptide was acetylated on the amino terminus to prevent rapid degradation by aminopeptidases (Pontremoli et al., 1980) and to radioactively label the peptide. A 40-μL aliquot of a 25 mM aqueous solution of imide hexapeptide was added to 40 μL of 3.0 M sodium acetate, pH 7.3 (pH adjusted by addition of glacial acetic acid), in a 1.5-mL polyethylene tube maintained at 0 °C with an ice bath. To start the acetylation reaction, 0.94 μL of a 20% (v/v) solution of acetic anhydride (reagent grade, Aldrich) in benzene (SpectrAR grade, Mallinckrodt) was added to the imide solution. This constitutes a 2-fold molar excess of acetic anhydride over imide. The reaction was allowed to proceed for 10 min after which it was quenched by the addition of 130 μL of 10% (w/v) trifluoroacetic acid. The reaction mixtures were partially dried by a gentle stream of $N_2$ flowing over the mixture for 3 h and purified by HPLC as described above. The extinction coefficient of the purified peptide at 214 nm was observed to be 15 380 $M^{-1}$ $cm^{-1}$.

$^{14}$C-Radiolabeled peptide was prepared by adding 40 μL of a 0.25 mM aqueous solution of hexapeptide imide to 40 μL of 0.1 M $Na_3PO_4$, pH 7.4, at 4 °C. A 20-fold molar excess of [$^{14}$C]acetic anhydride (ICN Radiochemicals, sp act. 10 mCi $mmol^{-1}$) was then added as a 1% (v/v) solution in benzene, and the mixture was incubated for 10 min as described above.

The HPLC-purified *N*-acetyl imide hexapeptide was characterized by positive ion mass spectroscopy on a modified Kratos MS9 instrument operated by Dr. Dilip Sensharma at the UCLA Department of Chemistry and Biochemistry. A sample of approximately 20 μg was suspended in 20 μL of glycerol and applied to a room temperature stainless steel electrode that was bombarded by xenon atoms emitted at a gun voltage of 4–6 kV and a current of 1 mA. The major peak of *m/e* 667 corresponded to a parent ion mass of the sodium species (M + 23). There were several lower molecular weight peaks that were consistent with the theoretical fragmentation pattern of the acetylated hexapeptide imide (Schafer, 1983). The product was also characterized by its electrophoretic mobility by thin-layer electrophoresis. A 5-nmol sample was applied to the midline of a 20-cm Eastman cellulose thin-layer sheet along with the unacetylated hexapeptide, tyrosine, and aspartate. Electrophoresis was performed in a buffer of 2% pyridine–0.95% acetic acid (pH 5.2) at 400 V for 30 min. Peptides and amino acids were detected by Pauly stain spray, which detects tyrosine residues (Smith, 1960). Unacetylated peptides and amino acids were also detected by ninhydrin spray. The *N*-acetyl imide hexapeptide migrated 1.5 cm toward the anode, aspartate migrated 5 cm toward the anode, the unacetylated imide hexapeptide migrated 1 cm toward the cathode, and tyrosine migrated 0.8 cm toward the cathode.

*N-Acetyl Aspartyl Hexapeptide and N-Acetyl Isoaspartyl Hexapeptide.* The normal aspartyl and the isoaspartyl derivatives of the acetylated hexapeptide were obtained by mild base hydrolysis of the *N*-acetyl imide hexapeptide as described (Murray & Clarke, 1984). In a typical reaction *N*-acetyl imide hexapeptide was suspended in water, 2 volumes of 0.1 M sodium borate (pH 10) was added, and the mixture was incubated for 40 min at 37 °C. The hydrolysis reaction was quenched by the addition of 1 half-volume of 10% trifluoroacetic acid, and the products of the reaction (approximately 80% isoaspartyl peptide and 20% normal aspartyl peptide) were separated by the HPLC system described above.

*Amino Acid Analysis.* Peptides collected from HPLC were acid hydrolyzed with 6 M HCl (Pierce, constant boiling) for 1 h at 150 °C in vacuo. The resulting amino acids were reacted with *o*-phthalaldehyde essentially as described (Jones et al., 1981) and separated by a Waters Resolve reverse phase column (3.9 × 150 mm, 5 μm, spherical C18 particles) and a Gilson Model 121 fluorometer with bandwidth filters (309–395-nm excitation and 430–470-nm emission wavelengths). Peak areas of fluorescent amino acid derivatives were integrated by a Shimadzu C-R3A, and molar ratios of amino acids were calculated from fluorescence coefficients determined with amino acid standards (Pierce Standard H). Amino acid residues were also derivatized with phenyl isothiocyanate (Pierce) and analyzed at 254 nm by the Waters Pico-Tag protocol (Bidlingmeyer et al., 1984).

*Carboxypeptidase Y Digestion of Peptides.* Lyophilized carboxypeptidase Y [a preparation from bakers' yeast, Sigma, containing 80% sodium citrate buffer (pH 5.0) and 20% protein, 100 units (mg of protein)$^{-1}$] was dissolved in water to a protein concentration of 4 mg $mL^{-1}$ and approximately 16 mg (mL of sodium citrate buffer)$^{-1}$, pH 5.0. To 20 μL of 0.2 M sodium citrate (pH 6.0) was added 10 μL of a 0.1 mM tripeptide aqueous solution and 1 μL of the carboxypeptidase Y solution. The mixture was allowed to incubate at 37 °C for 75 min and was quenched by immersing the reaction vessel into an ice bath. The amino acid products of the digestion were analyzed by *o*-phthalaldehyde derivatization as described above.

*Preparation of Erythrocyte Cytosol.* Volunteer human blood was drawn into heparinized tubes and separated from nonerythroid cells by plasmagel (Laboratoire Roger Bellon,

[1] Abbreviations: DEAE, diethylaminoethyl; CBZ, carbobenzoxy; MEC, 4-methylcoumarin-7-amide; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; Ac, acetyl; Asu, aspartyl succinimide; TRH, thyrotropin releasing hormone; Tris, tris(hydroxymethyl)aminomethane.

Neuilly Sur Seine, France) separation. The plasmagel was mixed with the blood in a 1:3 ratio and allowed to settle at room temperature for 30 min. The plasmagel containing the nonerythroid cells (upper layer) was discarded, and the small amount of white buffy coat that remained with the erythrocytes was collected and removed as described (Frietag & Clarke, 1981; Murray & Clarke, 1984). To determine the amount of contaminating nonerythrocytes in the erythrocyte preparation, a representative preparation of cells was stained with Wright's stain, and no white cells were detected after viewing of approximately 150000 cells with a light microscope. Furthermore, a preparation of purified red blood cells was counted with a hemocytometer, and the frequency of white blood cell contamination was determined to be less than $10^{-5}$. The erythrocytes were lysed by freeze-thawing in 5 volumes of buffer A [5 mM sodium phosphate, 5 mM $Na_2EDTA$, 15 mM 2-mercaptoethanol, 10% (w/v) glycerol, pH 7.4] as described (Murray & Clarke, 1984). Membranes were removed by centrifugation at 37000*g* for 60 min, and the resulting cytosolic fraction was stored at −70 °C. Protein concentration was measured by the Bradford assay (Bradford, 1976).

*Fluorogenic Assay for Proline Endopeptidase Activity.* This assay is based on the ability of proline endopeptidase to cleave the fluorogenic substrate *N*-CBZ-Gly-Pro-4-methylcoumarin-7-amide and was done essentially as described by Yoshimoto et al. (1979). A 2-μL aliquot of the fluorogenic substrate was dissolved in dioxane and added to 98 μL of enzyme solution in buffer A, resulting in a final concentration of 12 μM substrate. The fluorescence of the 7-amino-4-methylcoumarin enzymatically released was monitored at 25 °C on an Aminco-Bowman spectrofluorometer with an excitation wavelength of 370 nm and an emission wavelength of 440 nm. The assay was quantitated by use of a 12 μM standard solution of 7-amino-4-methylcoumarin (Sigma). This assay could only be performed on hemoglobin-free samples because the fluorescence was quenched by heme absorbance.

*HPLC Assay for Tripeptide Production.* This assay method is based on the fact that *N*-acetyl-Val-Tyr-Pro has a shorter retention time than the *N*-acetyl imide hexapeptide on the reverse-phase HPLC system we used. Erythrocyte cytosol or purified extracts containing post-proline endopeptidase were incubated with a final concentration of 20 μM acetylated imide hexapeptide at 37 °C. The reaction was quenched by the addition of an equal volume of 10% trifluoroacetic acid (w/v), and the mixture was centrifuged at 8000*g* for 1 min. The supernatant was injected onto the HPLC system described above and the products were eluted with a linear gradient beginning at 10% solvent B and ending at 25% solvent B over a 35-min period. This assay could also be used with the *N*-acetyl isoaspartyl hexapeptide substrate but not with the *N*-acetyl aspartyl hexapeptide substrate because this peptide had approximately the same retention time as the tripeptide.

*Thin-Layer Chromatography Assay for Tripeptide Production.* *N*-[$^{14}$C]Acetyl hexapeptide substrates were incubated with erythrocyte cytosol and applied to the HPLC system as described above. The eluant from the HPLC column was collected in 1-mL fractions. Half of each fraction was added to 10 mL of scintillation cocktail (ACS II, Amersham), and radioactivity was measured by liquid scintillation counting. Fractions that showed radioactivity above background were pooled together, lyophilized, and resuspended in 10 μL of methanol or water. The resuspended material was spotted on a silica gel plate (EM Science, West Germany, 0.2-mm thickness), and the peptides were separated by 10:4 2-butanol–water solvent. After the solvent had reached the top of the gel plate, it was air-dried overnight and exposed to Kodak XAR-5 X-ray film. The normal peptides and isopeptides had an $R_f$ value of 0.10; the imide and tripeptide had $R_f$ values of 0.28 and 0.36, respectively. Amounts of peptide were quantified by densitometry measurements of the autoradiograph on a Quick Scan R&D densitometer (Helena Laboratories).

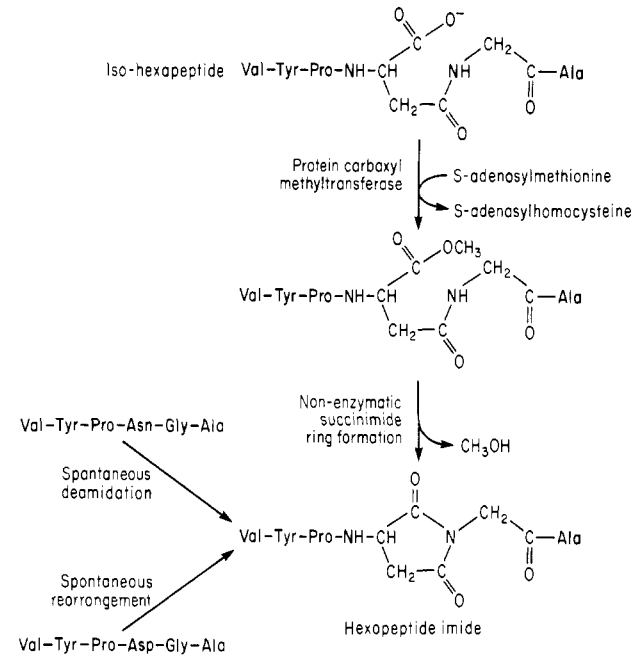


FIGURE 1: Three postulated reaction pathways resulting in protein succinimide formation. Aspartyl succinimides have been shown to form from both aspartyl and asparagine residues by a spontaneous intramolecular cyclization reaction (Geiger & Clarke, 1987). L-Isoaspartyl residues can also be converted to aspartyl succinimides through a two-step process. In the first step the recognition of an L-isoaspartyl residue by a protein carboxyl methyltransferase present in a variety of tissues results in the enzymatic methylation of the free α-carboxyl group (Aswad, 1984; Murray & Clarke, 1984). In the subsequent step, the methyl group can be nonenzymatically displaced by a similar intramolecular reaction as described above to generate the aspartyl succinimide (Murray & Clarke, 1986). The succinimide residue is prone to rapid racemization and hydrolysis reactions, leading to the production of D- and L-isoaspartyl and aspartyl residues (Geiger & Clarke, 1987). Examples of these reactions with hexapeptide sequences derived from adrenocorticotropic hormone 22–27 are shown.

*Purification of Erythrocyte Tripeptide-Producing Activity by Sephadex G-75 Gel Filtration Chromatography.* A 10-mL aliquot of cytosol was applied to a 92 × 2.5 cm Sephadex G-75 column (Pharmacia, superfine) equilibrated with buffer A at a flow rate of 25 mL $h^{-1}$. The peak of tripeptide-producing activity from hexapeptide was found to elute between 200 and 250 mL of elution volume. After concentration on an Amicon filtration concentrator equipped with a Diaflo membrane with a molecular weight cutoff of 10000, the final enzyme solution of 7.6 mL had a specific activity of 8 units $mg^{-1}$ (1 unit = 1 pmol of tripeptide produced per minute).

## RESULTS

*Detection of an Enzymatic Activity in Human Erythrocytes That Cleaves N-Ac-Val-Tyr-Pro-Asu-Gly-Ala To Produce N-Ac-Val-Tyr-Pro.* To determine the metabolic fate of proteins containing aspartyl succinimide residues, we used peptides to probe cleavage at these sites by enzymes present in red blood cells. Aspartyl succinimide residues are intermediates in the spontaneous degradation of proteins at asparagine and aspartyl residues as well as in the metabolism of enzymatically methylated L-isoaspartyl residues (Figure 1). Preliminary studies

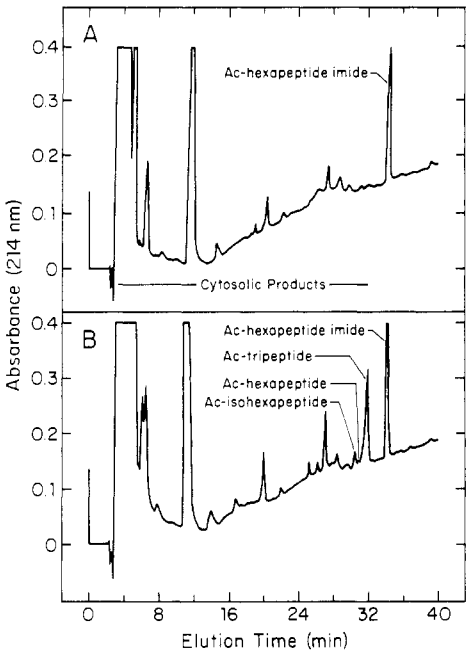


FIGURE 2: Degradation of *N*-Ac-Val-Tyr-Pro-Asu-Gly-Ala (*N*-acetyl imide hexapeptide) in cytosolic extracts of human erythrocytes. A 300-μL aliquot of erythrocyte cytosol [70 mg (ml of protein)$^{-1}$] was incubated with 200 μL of 0.2 M sodium HEPES buffer containing 10 nmol of *N*-acetyl imide hexapeptide (pH 7.4). (A) HPLC chromatograph of 40 μL of the incubation mixture quenched immediately after the addition of the cytosolic extract by 500 μL of 10% (w/v) trifluoroacetic acid. (B) HPLC chromatograph of 90 μL of the mixture incubated for 30 min at 37 °C before quenching. The identity of the *N*-Ac-Val-Tyr-Pro (tripeptide) was confirmed by amino acid analysis. The two peaks that elute just prior to the tripeptide were identified as *N*-acetyl isoaspartyl hexapeptide and *N*-acetyl aspartyl hexapeptide and were probably produced by the nonenzymatic hydrolysis of the *N*-acetyl imide hexapeptide (Murray & Clarke, 1984). These identifications were confirmed by rechromatography of the material in these peaks with known standards (see Materials and Methods).

on the metabolism of the synthetic peptide Val-Tyr-Pro-Asu-Gly-Ala in human erythrocyte cytosol were complicated by the rapid degradation of both the succinimide and the normal aspartyl form of the peptide (Murray & Clarke, 1986). Since this degradation is likely due to aminopeptidases (Pontremoli et al., 1980; Vandenberg et al., 1985) that would not be expected to be active on proteins containing internal succinimides, we decided to chemically block the amino terminus of the hexapeptide imide with an acetyl group. This chemical modification also allowed us to introduce a radiolabel into this peptide (see Materials and Methods). Because no carboxypeptidase activity has been found in erythrocytes (Pontremoli et al., 1980; Vandenberg et al., 1985), it was not necessary to chemically block the carboxyl terminus.

When the *N*-acetyl imide hexapeptide was incubated in human erythrocyte cytosol, a major UV-absorbing product was detected with an elution time on HPLC that was approximately 2 min less than that of the initial imide peptide (Figure 2). Since the primary chromophore of the imide peptide is the tyrosine residue, this product was probably a tyrosine-containing fragment. Control experiments using erythrocyte cytosol without added substrate showed that this new peak was not an endogenous product from the cytosol itself. The structure of the product isolated from HPLC was established as *N*-Ac-Val-Tyr-Pro. After acid hydrolysis the relative amino acid molar composition of the product was $Val_{0.81}Tyr_{1.00}Asp_0Gly_0Ala_0$ as compared to a composition of $Val_{1.08}Tyr_{1.15}Asp_{1.00}Gly_{0.94}Ala_{1.11}$ for the unreacted *N*-acetyl

Table I: pH Dependence of Human Erythrocyte Tripeptide-Producing Activity

| buffer (0.14 M)[a] | final pH | relative activity[b] |
|---|---|---|
| glycine | 2.42 | 0 |
| acetate | 4.53 | 0 |
| citrate | 5.87 | 0.20 |
| HEPES | 7.33 | 1.00 |
| Tris | 7.85 | 0.73 |
| borate | 9.04 | 0 |
| HEPES | 7.33 | 0[c] |

[a] Sodium salt of buffering species was used for all buffers except Tris, where the chloride salt was used. [b] A 10-μL aliquot of the Sephadex G-75 partially purified enzyme preparation was added to 35 μL of the indicated buffer with a final *N*-acetyl imide hexapeptide concentration of 10 μM. After 15 min at 37 °C the reaction was quenched by the addition of 50 μL of 10% (w/v) trifluoroacetic acid, and the tripeptide produced was measured by the HPLC assay. An activity of 1.0 represents 5.6% cleavage of the imide under these conditions. [c] The enzyme preparation was heated to 85 °C for 30 min prior to addition to the substrate/buffer solution.

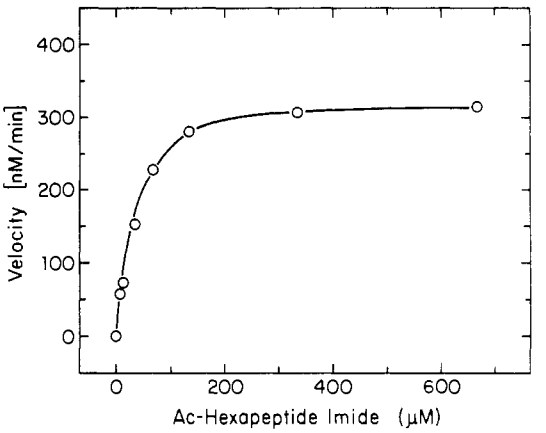


FIGURE 3: Effect of *N*-acetyl imide hexapeptide concentration on the initial rate of tripeptide production. Enzyme obtained by partial purification of erythrocyte cytosol on Sephadex G-75 chromatography (see Materials and Methods) was added as a 50-μL aliquot (35 mg/mL) to 100 μL of 0.2 M sodium HEPES buffer (pH 7.4) containing the *N*-acetyl imide hexapeptide concentration indicated. The reaction was allowed to proceed for 15 min at 37 °C and then quenched by the addition of 100 μL of 10% trifluoroacetic acid. The product was identified by the HPLC assay. The solid line represents the results expected for Michaelis–Menten kinetics with a $K_m$ of 36 μM and a $V_{max}$ of 276 pmol min$^{-1}$ (mg of protein)$^{-1}$.

imide hexapeptide. When the product was treated with carboxypeptidase Y, only free tyrosine was detected by *o*-phthalaldehyde amino acid analysis, suggesting that the peptide contained an *N*-acetylvaline residue that could not be cleaved by carboxypeptidase Y. Since the *o*-phthalaldehyde procedure does not detect proline, we performed phenyl isothiocyanate (PITC) derivatization of the acid-hydrolyzed peptide product and observed that it contained stoichiometric amounts of valine, tyrosine, and proline. In both types of analysis the absence of glycine, alanine, and aspartate was consistent with cleavage of the imide peptide between the proline and succinimide residue to give a tripeptide product.

*Characterization of Tripeptide-Producing Activity.* We partially purified this activity by Sephadex G-75 gel filtration chromatography. The elution position was consistent with a molecular weight between 60 000 and 100 000. The optimal pH for tripeptide-producing activity of this preparation ranged between pH 6 and pH 8, and heating it at 85 °C for 30 min completely abolished this activity (Table I). Tripeptide production catalyzed by the G-75 fraction was saturable with the *N*-acetyl imide substrate, and a $K_m$ of 36 μM was observed (Figure 3). Since the activity was heat-labile, displayed

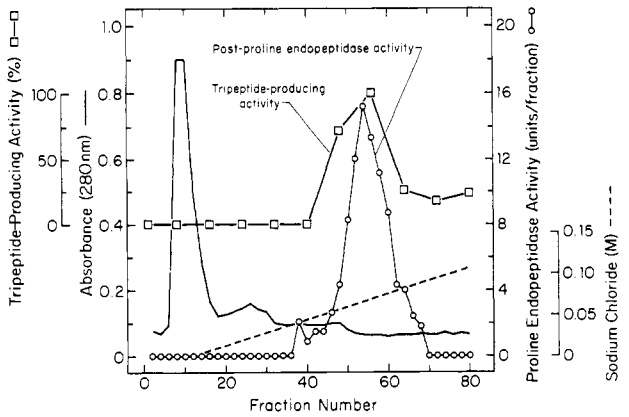


FIGURE 4: Copurification of tripeptide-producing activity and post-proline endopeptidase from human erythrocyte cytosol during DEAE-cellulose chromatography. Erythrocyte cytosol [4 mL, 85 mg (mL of protein)$^{-1}$] was applied to a 2.5 × 16 cm DEAE-cellulose column (Whatman DE-52 resin) equilibrated with 5 mM sodium phosphate, 5 mM $Na_2EDTA$, 15 mM 2-mercaptoethanol, and 10% (w/v) glycerol, pH 7.4, at 4 °C. The enzyme was eluted with an increasing linear gradient of buffer B [5 mM sodium phosphate, 5 mM $Na_2EDTA$, 15 mM 2-mercaptoethanol, 250 mM sodium chloride, 10% (w/v) glycerol, pH 7.4] with a flow rate of 42 ml h$^{-1}$. Fractions of 6.3 mL each were collected. Tripeptide-producing activity (open squares) was measured by incubating indicated fractions with 20 μM *N*-acetyl imide hexapeptide for 30 min at 37 °C, quenching with a half-volume of 10% trifluoroacetic acid, and determining the amount of tripeptide produced by the HPLC assay (see Materials and Methods). Post-proline endopeptidase activity (open circles) was measured by incubating the indicated fractions with 12 μM *N*-CBZ-Gly-Pro-MEC and determining the amount of relative fluorescence intensity produced over a 10-min period at room temperature (1 unit = 1 nmol of 7-amino-4-methylcoumarin produced per minute).

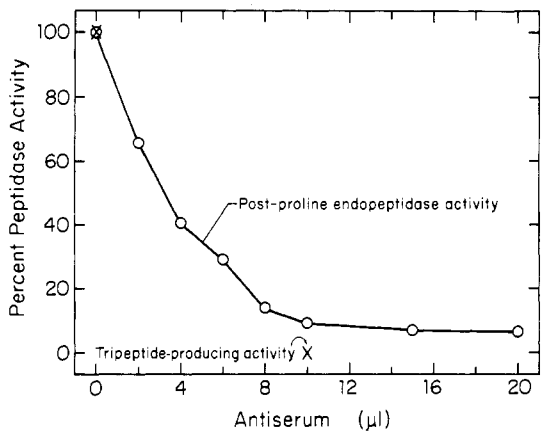


FIGURE 5: Inhibition of tripeptide-producing activity and post-proline endopeptidase by antiserum to rat brain post-proline endopeptidase. Indicated aliquots of 10 mg ml$^{-1}$ antiserum solution were incubated with 100 μL of DEAE-cellulose-purified human erythrocyte post-proline endopeptidase for 1 h at 0 °C. To measure post-proline endopeptidase activity, 2 μL of 0.6 mM *N*-CBZ-Gly-Pro-MEC was added to 98 μL of the enzyme/antiserum mixture, and the relative fluorescent intensity was monitored at room temperature for 10 min. The tripeptide-producing activity was measured by adding *N*-[$^{14}$C]acetyl imide hexapeptide to a final concentration of 20 μM and incubating the mixture for 1 h at 37 °C. The reaction was quenched by immersing the reaction vessel into dry ice, and the products were separated by HPLC and TLC. The amount of inhibition was quantitated by densitometry measurements of the autoradiograph of the TLC plate.

saturation kinetics, and had maximum activity near neutral pH, it appeared that tripeptide production was catalyzed by an enzyme active at the physiological pH of erythrocytes.

*Partial Purification of Tripeptide-Producing Activity and Identification as a Post-Proline Endopeptidase.* When erythrocyte cytosol was fractionated by DEAE-cellulose chromatography, tripeptide-producing activity was found to separate from the bulk of the protein (Figure 4). Because the peptide bond cleaved in this peptide was Pro–Asu, we decided to see if a post-proline endopeptidase activity was responsible by testing each of the DEAE-cellulose column fractions for post-proline endopeptidase activity with the fluorogenic substrate for this enzyme, *N*-CBZ-Gly-Pro-4-methylcoumarin-7-amide. We found that the two activities coeluted from the DEAE-cellulose column under these conditions, suggesting that the tripeptide-producing activity may in fact represent post-proline endopeptidase (Figure 4).

To test if the enzyme catalyzing the peptidase activity was immunologically related to post-proline endopeptidase, rabbit antisera against rat brain post-proline endopeptidase [obtained from Drs. Jack E. Dixon and Philip C. Andrews at Purdue University and prepared as described (Andrews et al., 1982)] was used to attempt to inhibit post-proline endopeptidase activity as well as the tripeptide-producing activity (Figure 5). We found that the erythrocyte post-proline endopeptidase activity was inhibited by the antiserum, suggesting that the human erythrocyte and rat brain enzymes are immunologically related. Furthermore, the antiserum also inhibited the tripeptide-producing activity, giving further evidence to the proposal that post-proline endopeptidase and the tripeptide-producing enzyme are the same.

When the DEAE-cellulose-purified material was applied to a Sephadex G-75 gel filtration column standardized with Dextran Blue (void volume), ovalbumin ($M_r$ 44 000), and cytochrome *c* ($M_r$ 12 500), we determined the native molecular weight of the human post-proline endopeptidase to be approximately 79 000. Rat brain post-proline endopeptidase was previously shown to have a $M_r$ of 73 000 (Rupnow et al., 1979), the corresponding enzyme from bovine pituitary gland has a $M_r$ of 76 000 (Knisatschek & Bauer, 1979), and the post-proline endopeptidase isolated from pig kidney has a molecular weight of approximately 70 000 (Hauzer et al., 1984). Thus, the molecular weight of post-proline endopeptidase from these mammalian tissues appears to be similar.

The previously characterized post-proline endopeptidases are serine proteases which can be inhibited by diisopropyl fluorophosphate (DFP) (Yoshimoto et al., 1977; Andrews et al., 1980). To determine if this was also the case with the human erythrocyte enzyme, and at the same time covalently label it at the active site, we incubated DEAE-cellulose-purified enzyme incubated with [$^3$H]DFP (Figure 6). We observed that this treatment inhibited post-proline endopeptidase activity by 72%. We were able to separate the labeled enzyme from the unreacted [$^3$H]DFP on a Sephadex G-75 column and then fractionated the polypeptides by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 6). We found that there was only one [$^3$H]DFP-radiolabeled polypeptide band that corresponded to a relative molecular weight of 83 000. This was in good agreement with the molecular weight determined from gel filtration chromatography and showed that the native structure of the endopeptidase probably consists of a single polypeptide chain.

In order to further investigate the substrate specificity of the cleavage reaction, we followed tripeptide production using 20 μM *N*-acetyl imide hexapeptide as a substrate in the presence of competing peptide inhibitors. It was found that when the competitive inhibitor was 1 mM unacetylated aspartyl hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala), the activity was inhibited 72%; 1 mM *N*-CBZ-Gly-Pro-Leu-Gly resulted in 80% inhibition. However, there was no inhibition when an aspartyl succinimide containing peptide, such as Trp-Met-

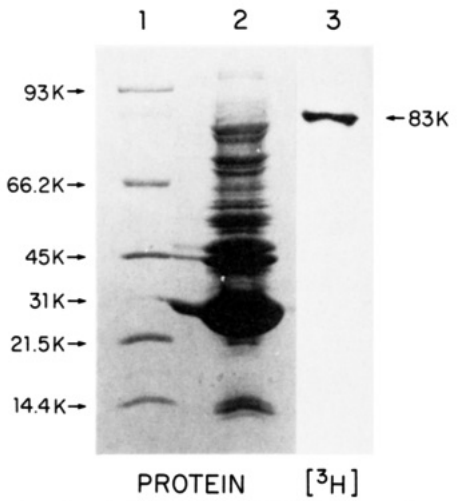


FIGURE 6: Covalent labeling of an 83 000-dalton polypeptide in a partially purified post-proline endopeptidase preparation with [$^3$H]diisopropyl fluorophosphate. DEAE-cellulose-purified enzyme was concentrated 10-fold by ultrafiltration (Amicon YM10 filter) and treated with 1.0 $\mu$L of 0.21 M [$^3$H]DFP (New England Nuclear, 4.7 Ci mmol$^{-1}$ specific activity) and allowed to incubate for 1 h at room temperature and then for another 20 h at 4 °C. Unreacted [$^3$H]DFP was separated from radioactively labeled protein by chromatography on a 1.5 × 88 cm Sephadex G-75 gel filtration column equilibrated with a buffer consisting of 5 mM sodium phosphate, 5 mM $Na_2EDTA$, 15 mM 2-mercaptoethanol, and 10% (w/v) glycerol, pH 7.4, at 4 °C. Fractions eluting from 56 to 88 mL were pooled and concentrated to 1 mL on a YM10 Diaflo filter. The concentrate was dialyzed against water overnight at 4 °C and then partially lyophilized to a final volume of 55 $\mu$L. Half of this material (11 000 cpm) was electrophoresed on a 10% cross-linked polyacrylamide gel containing sodium dodecyl sulfate (Laemmli, 1970). After staining, the gel was soaked in a fluorescent-activating solution (EN$^3$HANCE, New England Nuclear), dried, and exposed to Kodak XAR-5 X-ray film for 8 days at −70 °C: (lane 1) Coomassie stain of low molecular weight standards (Bio-Rad Laboratories); (lane 2), Coomassie stain of [$^3$H]DFP-modified post-proline endopeptidase enzyme preparation; (lane 3) autoradiograph of [$^3$H]DFP-modified post-proline endopeptidase enzyme preparation.

Table II: Tripeptide Formation Is Dependent on Configuration of the Aspartyl Residue in the Hexapeptide Substrate

| substrate | relative tripeptide-producing activity[a] |
|---|---|
| *N*-Ac-Val-Tyr-Pro-Asu-Gly-Ala | 1.00 |
| *N*-Ac-Val-Tyr-Pro-D-Asu-Gly-Ala | 1.00 |
| *N*-Ac-Val-Tyr-Pro-isoAsp-Gly-Ala | 0.46 |
| *N*-Ac-Val-Tyr-Pro-D-isoAsp-Gly-Ala | 0.20 |

[a] Substrate was incubated at a concentration of 20 $\mu$M with erythrocyte cytosol [42 mg (mL of protein)$^{-1}$] at pH 7.4 at 37 °C. After 90 min the reaction was quenched with an equal volume of 10% trifluoroacetic acid, and tripeptide was quantitated by the HPLC assay. A relative activity of 1.00 represents a conversion of 26.2% of the original hexapeptide to the tripeptide.

Asu-Phe-$NH_2$ was used as the competitive inhibitor at a concentration of 0.5 mM. These results provide confirming evidence that the peptide cleaving activity recognizes the proline residue of the peptide.

*Specificity of the Post-Proline Endopeptidase for Proline–Aspartyl Derivatives.* To test whether the succinimide portion of the *N*-acetyl imide hexapeptide influenced the rate of peptide cleavage, the acetylated L-aspartyl succinimide, D-aspartyl succinimide, L-isoaspartyl, and D-isoaspartyl hexapeptides were prepared and incubated separately with erythrocyte cytosol as described in the legend of Figure 2. L and D refer to the stereoconfiguration the $\alpha$-carbon of the aspartyl residue. As shown in Table II, the rate of L-isoaspartyl hexapeptide cleavage was less than half the rate of

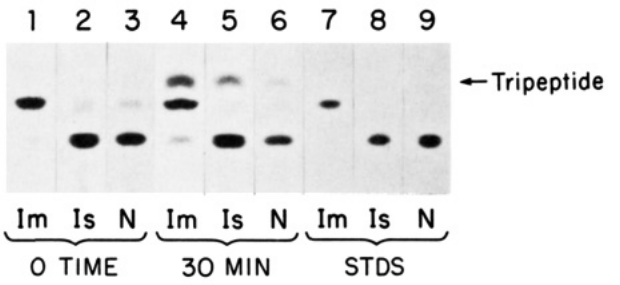


FIGURE 7: Production of *N*-Ac-Val-Tyr-Pro from the *N*-acetyl imide hexapeptide, the *N*-acetyl isoaspartyl hexapeptide, and the *N*-acetyl aspartyl hexapeptide. *N*-[$^{14}$C]Acetyl hexapeptides (Im = imide hexapeptide, Is = isoaspartyl hexapeptide, N = aspartyl hexapeptide) were separately incubated at a final concentration of 20 $\mu$M in 40 $\mu$L of erythrocyte cytosol [70 mg (mL of protein)$^{-1}$] and 160 $\mu$L of 0.2 M sodium HEPES buffer, pH 7.4, at 37 °C. The reactions were quenched by the addition of 2 volumes of 10% (w/v) trifluoroacetic acid. The products were purified and separated on HPLC and TLC (see Materials and Methods): (lanes 1–3) reaction mixtures were immediately quenched after the addition of cytosol; (lanes 4–6) reaction mixtures were incubated for 30 min before quenching; (lane 7) *N*-Ac-Val-Tyr-Pro-Asu-Gly-Ala standard 100 pmol); (lane 8) *N*-Ac-Val-Tyr-Pro-isoAsp-Gly-Ala standard (100 pmol); (lane 9) *N*-Ac-Val-Tyr-Pro-Asp-Gly-Ala standard (100 pmol).

the L-aspartyl succinimide hexapeptide cleavage, suggesting that the post-proline endopeptidase prefers the imide substrate over the isoaspartyl substrate. The D-aspartyl succinimide and the L-aspartyl succinimide served equally well as substrates for the peptidase activity.

We also wanted to test whether the *N*-acetyl normal aspartyl hexapeptide was a substrate for tripeptide production. This was not possible with the HPLC assay alone because the tripeptide and *N*-acetyl hexapeptide had approximately the same elution position (see Figure 2B). Therefore, a thin-layer chromatography procedure was developed to separate these two peptides. As observed in Figure 7, the L-aspartyl succinimide, L-isoaspartyl, and L-aspartyl hexapeptides all produced the tripeptide product. However, the L-isoaspartyl cleavage rate was approximately 50% that of the L-aspartyl succinimide cleavage rate while the L-aspartyl cleavage rate was about 10% that of the imide. Thus, it appears that the aspartyl succinimide containing peptide is the preferred substrate for tripeptide production.

## Discussion

Proteins containing atypical amino acids such as D- and L-aspartyl succinimide and D- and L-isoaspartyl residues may be metabolized in cells by proteolytic degradation reactions. In this study we have utilized synthetic hexapeptides to compare the specificities of erythrocyte endopeptidases with substrates containing these residues. For *N*-Ac-Val-Tyr-Pro-(Asp)-Gly-Ala derivatives, we have found that the major route of metabolism is the cleavage of the proline–(aspartyl) bond to produce the tripeptide *N*-Ac-Val-Tyr-Pro.

The tripeptide-producing activity appears to catalyzed by a post-proline endopeptidase. This activity can be inhibited by proline-containing peptides but not by peptides that do not contain proline. We have demonstrated that the tripeptide-producing activity and an activity that cleaves *N*-CBZ-Gly-Pro-4-methylcoumarin-7-amide, a substrate for post-proline endopeptidase (Yoshimoto et al., 1979), coelute on DEAE-cellulose chromatography and are both inhibited by antisera raised against rat brain post-proline endopeptidase. Furthermore, the erythrocyte post-proline endopeptidase activity was inhibited 72% and covalently labeled by [$^3$H]diisopropyl fluorophosphate, characteristic of post-proline endopeptidases

Table III: Comparison of Affinity of Post-Proline Endopeptidase Activity for Various Peptide Substrates

| structure and site of cleavage[a] | $K_m$ ($\mu$M) | ref |
|---|---|---|
| pGlu-His-Pro-$NH_2$ | 4100 | Andrews et al., 1980 |
| Bz-Gly-Pro-Leu-Gly | 1370 | Nomura, 1986 |
| *N*-CBZ-Gly-Pro-Leu-Gly | 540 | Nomura, 1986 |
| *N*-CBZ-Gly-Pro-Leu-Gly | 60 | Koida & Walker, 1976 |
| *N*-Ac-Val-Tyr-Pro-Asu-Gly-Ala | 36 | this study |
| pGlu-$N^{im}$-benzyl-His-Pro-2-NNap | 34 | Rupnow et al., 1979 |
| *N*-CBZ-Gly-Pro-2-NNap | 20 | Yoshimoto et al., 1979 |
| pGlu-His-Pro-2-NNap | 15 | Knisatschek et al., 1980 |
| *N*-CBZ-Gly-Pro-2-NNap | 14 | Knisatschek et al., 1980 |
| *N*-CBZ-His-Pro-2-NNap | 9.5 | Knisatschek et al., 1980 |

[a] pGlu, L-pyroglutamyl; $N^{im}$, *N*-imidazolyl; 2-NNap, $\beta$-naphthylamide.

isolated from other tissue sources (Yoshimoto et al., 1977; Andrews et al., 1980). All of this evidence is consistent with the identification of the tripeptide-producing activity as an erythrocytic form of post-proline endopeptidase.

Post-proline endopeptidase was first detected in human uterus tissue as an activity that cleaved oxytocin at the Pro–Leu bond (Walter et al., 1971). The same activity was detected in rat brain (Rupnow et al., 1979), lamb kidney (Koida & Walter, 1976), and human body fluids (Yoshimoto et al., 1979). This paper is the first report of post-proline endopeptidase from human erythrocytes. Furthermore, the enzyme has been shown to be active on a variety of synthetic and natural peptide substrates (Table III; Taylor & Dixon, 1980; Orlowski, 1983) but does not appear to cleave large denatured polypeptides (Taylor & Dixon, 1980).

Another erythrocyte peptidase that may produce the tripeptide from *N*-Ac-Val-Tyr-Pro-(Asp)-Gly-Ala hexapeptides is tripeptidyl peptidase II (Balow et al., 1986). This enzyme is an aminopeptidase with a molecular weight of 135 000, a pH optimum of 7.5, and an activity that can be inhibited by phenylmethanesulfonyl fluoride (PMSF). We are confident that this activity is not responsible for tripeptide production because the enzyme described in this study is not inhibited by PMSF (data not shown), has a molecular weight determined by gel filtration chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis to be approximately 80 000, and has the capability to cleave a blocked *N*-terminal peptide while the tripeptidyl peptidase II requires a free N-terminus in order to cleave its substrate.

Surprisingly, the erythrocyte prolidase has a greater specificity for a Pro-Asp succinimide sequence rather than a normal Pro-Asp sequence (Figure 7). This does not appear to be simply an effect of the negative charge of the residue following the proline because the Pro-isoAsp sequence is also cleaved at a faster rate than the normal peptide. This greater specificity for the altered peptide reveals that the active site of the enzyme does not require a normal amino acid after the proline. It should be noted that three different synthetic chromophoric peptides with non amino acid residues on the carboxyl side of the scissle bond exhibit $K_m$'s that are lower than the $K_m$'s reported for peptides with normal amino acid residues in the same position (Table III). It is also interesting to note that the post-proline endopeptidase cleaves the L- and D-Asu stereoisomers at the same rate. When proline peptides were compared for their ability to act as substrates for lamb kidney post-proline endopeptidase, there was a significant decrease in the rate of cleavage of the Pro–X bond if X was a D-amino acid. For example, the efficiency of cleavage of *N*-CBZ-Gly-Pro-D-Ala (measured as $K_{cat}/K_m$) was 3.2% of the corresponding L peptide, and in the case of *N*-CBZ-Gly-Pro-D-Leu the efficiency was 1.4% of the corresponding L peptide (Walter & Yoshimoto, 1978). The erythrocyte peptidase apparently loses this stereospecificity if the aspartyl residue is transformed into a succinimide, suggesting that the stereospecificity of the peptidase for the amino acid residue following the proline can be lost when an altered amino acid is present.

In previous work it has been shown that aspartyl succinimide and isoaspartyl peptides can arise from deamidation of asparaginyl residues for both peptides as well as proteins at physiological pH (Geiger & Clarke, 1987; Di Donato et al., 1986). When these abnormal residues occur on the carboxyl side of proline, it appears to make that substrate more susceptible to cleavage by post-proline endopeptidase. Since the presence of D-aspartyl, D- and L-isoaspartyl, and D- and L-aspartyl succinimide residues might be expected to disrupt normal protein structure, the degradation of proteins containing these residues may be important in maintaining cellular functions.

## ACKNOWLEDGMENTS

We are grateful to Dr. Philip C. Andrews and Dr. Jack Dixon for supplying us with antisera to rat brain post-proline endopeptidase and to Dr. David Sigman for the use of his fluorometer.

**Registry No.** *N*-Ac-Val-Tyr-Pro-Asu-Gly-Ala, 110826-64-7; *N*-Ac-Val-Tyr-Pro-D-Asu-Gly-Ala, 110901-96-7; *N*-Ac-Val-Tyr-Pro-isoAsp-Gly-Ala, 110826-65-8; *N*-Ac-Val-Tyr-Pro-D-isoAsp-Gly-Ala, 111001-18-4; post-proline endopeptidase, 72162-84-6.

## REFERENCES

Ahern, T. J., & Klibanov, A. M. (1985) *Science (Washington, D.C.) 228*, 1280–1284.

Andrews, P. C., Hines, C. M., & Dixon, J. E. (1980) *Biochemistry 19*, 5494–5500.

Andrews, P. C., Minth, C. D., & Dixon, J. E. (1982) *J. Biol. Chem. 257*, 5861–5865.

Aswad, D. W. (1984) *J. Biol. Chem. 259*, 10714–10721.

Balow, R.-M., Tomkinson, B., Ragnarsson, U., & Zetterqvist, O. (1986) *J. Biol. Chem. 261*, 2409–2417.

Bidlingmeyer, B. A., Cohen, S. A., & Tarrin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brunauer, L. S., & Clarke, S. (1986) *J. Biol. Chem. 261*, 12538–12543.

Clarke, S. (1985) *Annu. Rev. Biochem. 54*, 479–506.

Di Donato, A., Galletti, P., & D'Alessio, G. (1986) *Biochemistry 25*, 8361–8368.

Freitag, C., & Clarke, S. (1981) *J. Biol. Chem. 256*, 6102–6108.

Geiger, T., & Clarke, S. (1987) *J. Biol. Chem. 262*, 785–794.

Hauzer, K., Jost, K., Low, M., & Polgar, L. (1984) *Acta Biochim. Biophys. Acac. Sci. Hung. 19*, 165–168.

Johnson, B. A., Murray, E. D., Jr., Clarke, S., Glass, D. B., & Aswad, D. W. (1987) *J. Biol. Chem. 262*, 5622–5629.

Jones, B. N., Pabo, S., & Stein, S. (1981) *J. Liq. Chromatogr. 4*, 565–586.

Knisatschek, H., & Bauer, K. (1979) *J. Biol. Chem. 254*, 10936–10943.

Knisatschek, H., Kleinkauf, H., & Bauer, K. (1980) *FEBS Lett. 111*, 157–161.

Koida, M., & Walter, R. (1976) *J. Biol. Chem. 251*, 7593–7599.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

McFadden, P. N., & Clarke, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2595–2599.

Murray, E. D., Jr., & Clarke, S. (1984) *J. Biol. Chem. 259*, 10722–10732.
Murray, E. D., Jr., & Clarke, S. (1986) *J. Biol. Chem. 261*, 306–312.
Nomura, K. (1986) *FEBS Lett. 209*, 235–237.
Orlowski, M. (1983) *Mol. Cell. Biochem. 52*, 49–74.
Pontremoli, S., Melloni, E., Salamino, F., Sparatore, S., Michetti, M., Benatti, U., Morelli, A., & De Flora, A. (1980) *Eur. J. Biochem. 110*, 421–430.
Rupnow, J. H., Taylor, W. L., & Dixon, J. E. (1979) *Biochemistry 18*, 1206–1212.
Schafer, W. (1983) in *Modern Methods in Protein Chemistry* (Tschesche, H., Ed.) pp 337–359, de Gruyter, New York.
Smith, I. (1960) in *Chromatographic and Electrophoretic Techniques* (Smith, I., Ed.) pp 291–307, Pitman, Bath, U.K.
Taylor, W. L., & Dixon, J. E. (1980) *Biochem. Biophys. Res. Commun. 94*, 9–15.
Vandenberg, J. I., King, G. F., & Kuchel, P. W. (1985) *Arch. Biochem. Biophys. 242*, 515–522.
Walter, R., & Yoshimoto, T. (1978) *Biochemistry 17*, 4139–4144.
Walter, R., Shlank, H., Glass, J. D., Schwartz, I. L., & Kerenyi, T. D. (1971) *Science (Washington, D.C.) 173*, 827–829.
Walter, R., Simmons, W. H., & Yoshimoto, T. (1980) *Mol. Cell. Biochem. 30*, 111–127.
Yoshimoto, T., Orlowski, R. C., & Walter R. (1977) *Biochemistry 16*, 2942–2948.
Yoshimoto, T., Kiyokazu, O., Walter, R., Koida, M., & Tsuru, D. (1979) *Biochim. Biophys. Acta 569*, 184–192.

# Synergistic Ligand Protection and Intermediates in the Denaturation of Extremely Thermophilic Glutamine Synthetase†

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*Received May 26, 1987; Revised Manuscript Received August 10, 1987*

ABSTRACT: Glutamine synthetase (GS) exists in the extreme thermophile *Bacillus caldolyticus* as two regulatory isoforms, E-I and E-II, produced as separate gene products. Thermostabilization of these isoforms was investigated by determining the kinetics of thermal denaturation as a function of temperature, compared to GS from mesophilic *Bacillus subtilis* (BSGS). Apoenzymes exhibited inherent thermostability of the order E-II > BSGS > E-I. Bound substrates and metal ions provided substantial thermostability, but ATP alone rendered each enzyme less thermostable. Analysis of activation parameters ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$) indicated that binding of ligands alters the kinetic barriers between native enzyme and the fully denatured state. The activation energies for certain species or complexes were unusually high: ca. 250 kcal/mol for apo-E-I and ca. 320 kcal/mol for the E-II·MnATP·Glu complex. In addition, strongly synergistic ligand stabilization was observed, especially for the thermophilic enzymes: that is, the sum of the changes in $\Delta G^{\ddagger}$ induced by addition of added L-glutamate or MnATP to apoenzyme was less than the $\Delta(\Delta G^{\ddagger})$ observed when both ligands were added together. The order of relative thermostabilities for fully complexed enzymes was E-II > E-I > BSGS. With few exceptions, the kinetic progress curves for thermal denaturation were biphasic, suggesting formation of a metastable intermediate in the pathway. Circular dichroism studies of equilibrium unfolding/refolding with E-I by guanidine hydrochloride (Gdn-HCl) also clearly indicated an intermediate in the pathway. Refolding of Gdn-HCl-denatured E-I was >90% reversible for this highly oligomeric ($n$ = 12) enzyme.

The concepts of cell thermophily and protein thermostability have intrigued microbiologists and biochemists for several decades. These related problems have proven to be much more complex and subtle than was originally conceived. Comparisons of the size, amino acid content, quaternary structures, and catalytic and regulatory properties of numerous thermophilic vs mesophilic enzymes indicated few obvious differences that correlated with thermostability (Singleton & Amelunxen, 1973; Biesecker et al., 1977; Amelunxen & Murdock, 1978; Wedler, 1978; Wedler & Merkler, 1985). Although it was originally thought that a single, general mechanism might be found to account for the increased thermostability of enzymes and proteins from thermophilic microorganisms (Koeffler, 1957), it now is apparent that this property can result from a variety of minor changes in protein bonding forces (Argos et al., 1979). Since the rate of denaturation at a given temperature is altered 10-fold by a change in $\Delta G^{\ddagger}$ of only ca. 1.4 kcal/mol, changing a single amino acid residue can dramatically alter the thermostability of a protein (Langridge, 1968; Yutani et al., 1978; Gruetter et al., 1979). Consequently, elucidation of factors responsible for thermostability of an enzyme or protein requires detailed physicochemical analysis.

Voordouw et al. (1976) have suggested that a major part of protein stability could be attributed to *activation parameters* (manifested by kinetic effects) rather than *thermodynamic* factors. Furthermore, it was proposed that such factors could be divided into two categories: (a) *intrinsic* properties of the folded polypeptide chain such as the stabilizing energies from

† Supported in part by NSF Grant DMB84-14025. This work was presented in part at the 13th IUB Meeting, August 1985.
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0006-2960/87/0426-7805$01.50/0 