Tertiary Structure in Deletion Analogues of Human β-Endorphin: Resistance to Leucine Aminopeptidase Action†

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ABSTRACT: The presence of tertiary structure in aqueous solutions of the amino-terminal nine- and seventeen-residue analogues of human β-endorphin has been further demonstrated by leucine aminopeptidase (LAP) cleavage of the amino-terminal tyrosine. The reactions were followed by difference absorption spectroscopy. While the amino-terminal pentapeptide showed no resistance to LAP action, the nonapeptide displayed definite resistance. The heptadecapeptide analogue was found to be completely refractory to LAP action under the conditions employed. Thermolysin cleavage of the Phe[4]–Met[5] bond in the longer analogues destroys the tertiary structure, not only removing the previously reported red shift in tyrosine absorption but also resulting in complete normalization to LAP digestion.

In previous studies (Nicolas et al., 1981; Bewley & Li, 1983) the naturally occurring, 31-residue opioid peptide β-endorphin (β-EP),[1] as well as several synthetic C-terminal deletion analogues, has been shown to exhibit tertiary structure in aqueous solution. The evidence for tertiary structure consists of a significant red shift in the UV absorption of the N-terminal Tyr[1] residue. This was discovered in the form of excess blue shifts in Tyr absorption produced during thermolysin digestion of the Phe[4]–Met[5] bond in these peptides. The extra red shift could be explained only by assuming folded forms involving the N-terminal Tyr[1] with residues C-terminal to Met[5]. Deletion analogues as short as β_h-EP-(1–17) and β_h-EP-(1–9) exhibited tertiary structures, although the pentapeptide β_h-EP-(1–5) did not.

Tertiary structure involving Tyr[1] has also been suggested by the observation (Gräf et al., 1977) that removal of Tyr[1] from β_h-EP with aminopeptidase M is only 70% as facile as removal of the same residue from β-EP-(1-5). More recently, it has been shown (Li & Chung, 1985) that although LAP readily removes Tyr[1] from β_h-EP-(1–5) in a 2-h digestion at 37 °C, β_h-EP is totally resistant.

In this study, the action of LAP and LAP combined with thermolysin on β_h-EP-(1–5), β_h-EP-(1–9), and β_h-EP-(1–17) have been followed by difference absorption spectroscopy. A resolution of the LAP-induced difference spectrum into its NH_2-group and COOH-group charge perturbations is also presented.

Materials and Methods

All synthetic procedures, chemical characterizations, and biological properties of β_h-EP-(1–5), β_h-EP-(1–9), β_h-EP-(1–17), and β_h-EP-(21–31) have been described (Li, 1981). Thermolysin, lot no. 73326, was obtained from Calbiochem-Behring. LAP, lot no. 54A5366, was from Cooper Biomedical, and α-chymotrypsin, lot no. H7N862, was from Worthington Biochemical Corp. *O*-Methyl-L-Tyr and L-Tyr-amide were obtained from Vega.

UV absorption spectra were recorded at 60 nm/min in quartz microcells on a Perkin-Elmer Model 552 spectrophotometer equipped with background correction and temperature control accessories. Zero-order, direct spectra were corrected for light scattering. All peptide solutions were centrifuged at 16000*g* for 15 min prior to use. Solute concentrations were determined from zero-order, direct spectra assuming E_M = 1420 M^{-1} cm^{-1} at 274.8 nm for all β_h-EP analogues and Tyr model compounds (Bewley & Li, 1983).

All enzyme digests were carried out at 25 °C in 0.05 M Tris-HCl and 0.01 M $MgCl_2$ buffer (pH 7.28) and followed by the difference spectral technique. With peptide in both sample and reference cells, the enzyme was added to the sample cell and difference spectra were recorded at appropriate time intervals. The molar concentration of all peptides and model compounds was held constant ($\sim 4 \times 10^{-4}$ M), and the same amount of each enzyme was used every time so that kinetics of the various digestions could be directly compared. The E/S ratio for LAP was 1/38 000 (mol/mol), and that for thermolysin was 1/40 000 (mol/mol). This small amount of enzyme contributed insignificantly to the absorptivity of the sample as shown in Figure 1 by only very slight departures from ideal base lines. The bond in β_h-EP-(1–17) that was most rapidly cleaved by thermolysin was identified, as previously described for thermolysin digests of β_h-EP (Bewley & Li, 1983). Chymotryptic digestion of β_h-EP-(21–31) was performed under the same conditions as for LAP and thermolysin. The E/S ratio was 1/2000 (mol/mol). Titration difference spectra of model compounds were obtained by addition of small volumes of concentrated HCl or saturated NaOH to the sample and corresponding amounts of buffer to the reference. A Radiometer Model 26 pH meter was used to measure all pH values.

Results

A family of difference spectra, generated during LAP digestion of β_h-EP-(1–5), is presented in Figure 1A. Each spectrum is characterized by a negative (blue-shift) maximum at 285.5 nm, a weaker negative maximum at 277.5 nm, and

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[1] Abbreviations: β-EP, β-endorphin; β_h-EP, human β-endorphin; LAP, leucine aminopeptidase; UV, ultraviolet; E/S, enzyme to substrate ratio; E_M, molar extinction coefficient; ΔE_M, change in molar extinction coefficient; α-NH_2, α-amino group; α-COOH, α-carboxyl group; α-COO^-, a fully ionized α-carboxylate group; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. The standard three-letter abbreviations are used for the amino acids.

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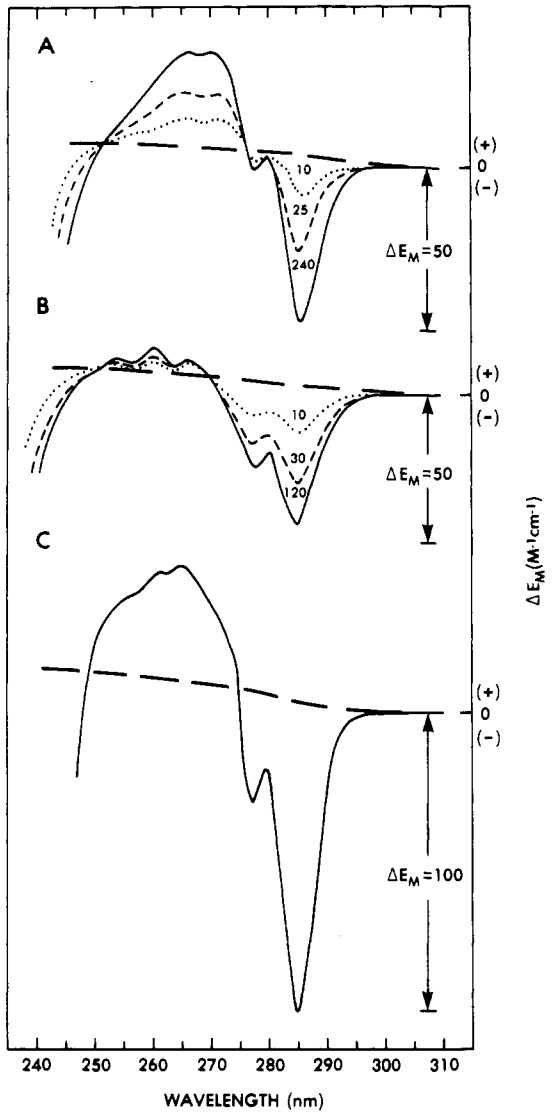

FIGURE 1: Difference absorption spectra generated during proteolysis of (A) β_h-EP-(1–5) with LAP, (B) β_h-EP-(1–17) with thermolysin, and (C) β_h-EP-(1–17) first with thermolysin, followed by LAP. The conditions of digestion are described in the test. In (A) and (B) the times of digestion (in minutes) are indicated above each spectrum. In (C), only the final difference spectrum is shown.

a broad, positive difference band between 276 and 252 nm. The areas under the positive bands are roughly equivalent to the areas above the two negative bands.

Figure 1B displays a similar family of difference spectra generated during thermolysin digestion of β_h-EP-(1–17). Two negative maxima again appear at 285 and 277.5 nm. However, in contrast to Figure 1A, the positive band between 270 and 252 nm is relatively weak and contains more fine structure than that in Figure 1A. The shape of this spectrum is identical with those previously published (Bewley & Li, 1983) for thermolysin digests of β_h-EP and various analogues.

Figure 1C is the final difference spectrum generated by initial digestion of β_h-EP-(1–17) with thermolysin, followed by further digestion with LAP. This spectrum is equivalent to the graphical sum of panels A and B of Figure 1.

Figure 2 presents the rate of appearance of the 285-nm, major, blue-shift peak during LAP digestions of β_h-EP-(1–5), β_h-EP-(1–9), and β_h-EP-(1–17). The final difference spectrum for β_h-EP-(1–5) shows a stable blue shift: $\Delta E_M = -52$ M^{-1} cm^{-1}. LAP digestion of β_h-EP-(1–9) provides a blue shift less than half as intense in the same 4-h time interval [24-h digestion will increase this to ~70–80% of the β_h-EP-(1–5) value]. Most notably, LAP added to β_h-EP-(1–17) shows no measurable blue shift within 24 h.

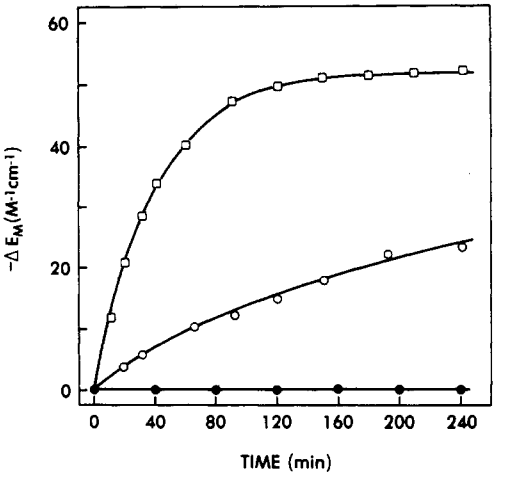

FIGURE 2: The rates of increase in ΔE_M for the major difference absorption peaks at 285 nm observed during LAP hydrolysis of (□) β_h-EP-(1–5), (○) β_h-EP-(1–9), and (●) β_h-EP-(1–17).

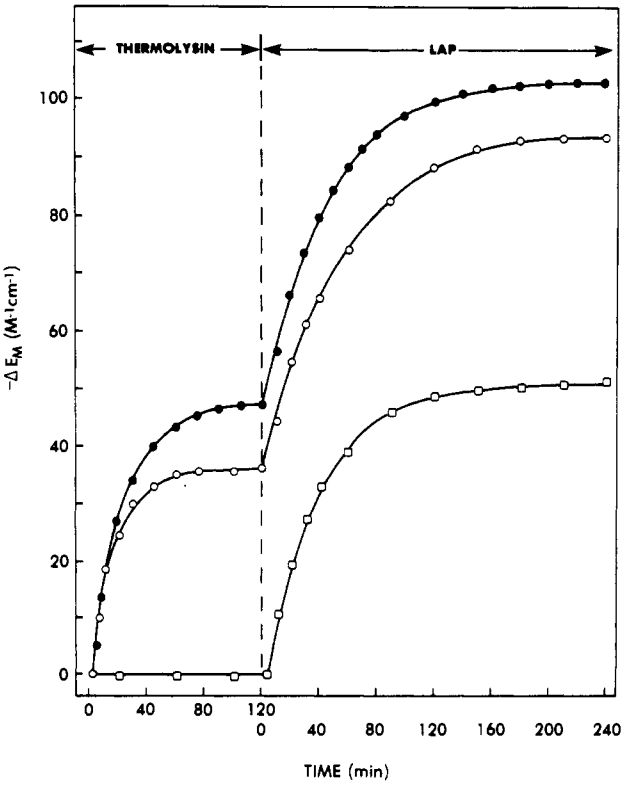

FIGURE 3: The rate of increase in ΔE_M for the major difference absorption peaks at 285 nm observed during thermolysin digestion, followed by LAP digestion of (□) β_h-(1–5), (○) β_h-EP-(1–9), and (●) β_h-EP-(1–17). The time axis indicates the zero times for both thermolysin and subsequent LAP additions.

Figure 3 presents the rate of appearance of the 285-nm blue-shift maxima obtained first by thermolysin digestion, followed by LAP digestion. In all cases the thermolysin reactions were allowed to continue until a stable difference spectrum was achieved before adding the LAP. Under these conditions, thermolysin will not cleave β_h-EP-(1–5) (Bewley & Li, 1983). The thermolysin digestions of β_h-EP-(1–9) and β_h-EP-(1–17) produce the same ΔE_M values at 285.5 nm previously indicated (Bewley & Li, 1983).

Subsequent addition of LAP to these digests then rapidly produces an additional blue shift, very similar to those seen

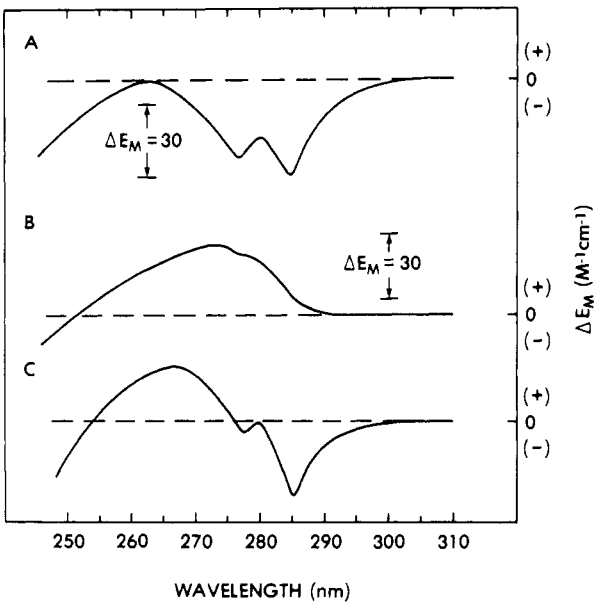

FIGURE 4: (A) Difference absorption spectrum generated during acid titration of L-Tyr-amide (pH 7.0–1.5). A similarly *shaped* difference spectrum is generated by acidic titration of *O*-methyl-L-Tyr (pH 11.5–7.28), although the ΔE_M is slightly different. In (B), the difference spectrum generated by chymotryptic digestion of β_h-EP-(21–31) at the bond between Tyr^{27} and Lys^{28} (pH 7.28) is shown. The graphical sum of the spectra in (A) and (B) is presented in (C).

in the LAP digestions of β_h-EP-(1–5) in either Figure 2 or Figure 3. The LAP-induced increase is nearly the same for all three peptides and falls on the same straight line (correlation coefficient = 0.95) when coplotted as first-order reactions (graph not shown). This line indicates a pseudo-first-order rate constant of 5.7×10^{-4} s^{-1} for production of free Tyr.

Figure 4A shows the difference spectrum generated by titration (protonation) of the α-NH_2 groups of L-Tyr-amide (sample at pH 1.5, reference at pH 7.0) and *O*-methyl-L-Tyr (sample at pH 7.28, reference at pH 11.5).[2] Either model, over the pH ranges cited, gives a similarly *shaped* difference spectrum, with two negative maxima and no positive ΔE_M. Complete protonation can produce a ΔE_M of up to -178 M^{-1} cm^{-1} (Wetlaufer, 1962). In contrast, chymotryptic cleavage (pH 7.28) of β_h-EP-(21–31) between Tyr^{27} and Lys^{28} results in a new, fully ionized α-COO^- group on Tyr^{27}, without any effect on its peptide-bound α-NH_2 group. The difference spectrum produced by this action (Figure 4B) is a broad, positive band from 285 to 250 nm, with no negative maxima.[3]

HPLC analyses of aliquots taken from a β_h-EP-(1–17) digest at 0, 3, 60, and 120 min after adding thermolysin (data not shown), clearly indicate that the most readily attacked bond is between Phe^4 and Met^5, as also shown for β_h-EP (Bewley & Li, 1983). This same bond remains the most thermolysin-susceptible site in the β_h-EP-(1–9) analogue.

Discussion

All of these blue-shift difference spectra have certain features in common. The stronger negative maximum near 285 nm and the weaker negative maximum near 277.5 nm are due to perturbations of the ring absorption of Tyr (Herskovits & Sorensen, 1968; Donovan, 1969; Wetlaufer, 1962). In addition, in those difference spectra that also show fine structure peaks between 270 and 250 nm, these originate in perturbations of Phe ring absorption (Wetlaufer, 1962; Donovan, 1969). For both chromophores, these perturbations arise from charge effects and/or alterations in hydrophobic shielding (Wetlaufer, 1962; Donovan, 1969). The possibility of hydrogen bonding of Tyr phenolic groups contributing to these spectra has been eliminated in previous studies (Nicolas et al., 1981; Bewley & Li, 1983).

As shown in panels A and B of Figure 1, thermolysin and LAP each produce a characteristic type of difference spectrum. These spectra can be understood in terms of the differing chemical actions of the two enzymes. LAP acts to cleave the Tyr^1–Gly^2 bond, releasing free Tyr. At pH 7.28, the free Tyr contains a new, fully ionized α-COO^-, as well as an α-NH_2 group whose state of ionization (pK_a) has been significantly altered.[4] These two phenomena independently produce charge perturbations on the Tyr ring absorption. Thus, LAP cleavage of an N-terminal Tyr is equivalent to a partial protonation of its α-NH_2 group (increase in pK_a) with a difference spectrum as in Figure 4A, *plus* formation of a new α-COO^- group with a difference spectrum as shown in Figure 4B. Figure 4C represents the graphical sum of panels A and B of Figure 4. Note that both the shape and ΔE_M values of Figure 4C are nearly identical with those of the difference spectrum shown in Figure 1A for LAP action on β_h-EP-(1–5). Therefore, in LAP-induced Tyr difference spectra, the negative maxima are due to charge perturbations from changing ionization of the α-NH_2 group, whereas the positive portion below $\sim$270 nm is largely due to perturbations caused by the new α-COO^- group.

The second type of difference spectrum encountered in this study is produced by thermolysin digestion (Figure 1B). This difference spectrum arises from thermolysin cleavage of the Phe^4–Met^5 bond in β_h-EP and all deletion analogues except β_h-EP-(1–5). This difference spectrum is characterized by relatively deep negative maxima due to perturbation of Tyr ring absorption, with only a very weak positive region from 270 to 250 nm that displays fine structure arising from charge perturbations of Phe ring absorption due to the new α-COO^- group on Phe^4 (Bewley & Li, 1983). Although the overall shape of the spectrum appears similar to that of Figure 4A, due to changing α-NH_2 ionization, a loss of weak hydrophobic shielding of Tyr^1 could produce a very similar difference spectrum (Herskovits & Sorensen, 1968). At present, we cannot unequivocally distinquish between these two mechanisms for the thermolysin-induced perturbations.

Having explained the origins of the difference spectra, we turn now to the rates of the two types of enzyme digestions as revealed by difference spectra. In the previous study (Bewley & Li, 1983) it was noted that there is no apparent difference in the rates of thermolysin cleavage of the Phe^4–Met^5 bond in β_h-EP and all deletion analogues except β_h-EP-(1–5). In contrast, this is not true of the LAP digests. β_h-EP-(1–5) is readily attacked by the enzyme with liberation of the N-terminal Tyr (Figure 2). However, at the same molar concentrations of substrate and enzyme, β_h-EP-(1–9) is only slowly hydrolyzed, while β_h-EP-(1–17) shows no evidence of reaction. These results are in agreement with data obtained from amino acid analyses of similar LAP digests of these analogues (Li & Chung, 1985) and extend the observations of Gräf et al. (1977). The data clearly indicate that as the

[2] Complete titration of the α-NH_2 group has been divided into two portions by using two different models in order to avoid simultaneous titrations of the α-COOH or phenolic groups.

[3] Note that the production of a fully ionized Tyr α-COO^- by cleavage of a secondary amide bond is *not* equivalent (spectrophotometrically) to titration of a free Tyr α-COOH.

[4] N-Terminal Tyr residues usually show an α-NH_2 pK_a of 7.3–7.8 (Martin et al., 1958; Perrin, 1965), while that of free Tyr is 9.11 (Dawson et al., 1959).

peptide chain is extended beyond Met[5], the N-terminus becomes progressively resistant to LAP, presumably through formation of some type of tertiary structure.

The presence of tertiary structure in the two relatively resistant peptides is further confirmed by the data in Figure 3. The analogues were first treated with thermolysin until a stable difference spectrum was established (1–2 h). If, after completion of the thermolysin reactions, LAP is added, all three undergo rapid blue shifts. Apparently, following thermolysin cleavage of the Phe[4]–Met[5] bond in the two longer peptides, the tertiary structure is lost, and removal of Tyr[1] by LAP becomes quite facile. If the LAP-induced increases in ΔE_M for the thermolysin-pretreated peptides are coplotted as first-order reactions, it is found that the three LAP reactions are now kinetically equivalent.

Registry No. β_h-EP-(1–9), 59481-79-7; β_h-EP-(1–17), 60893-02-9; β_h-EP-(1–5), 58569-55-4; LAP, 9001-61-0.

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Incorporation of the Purified Human Placental Insulin Receptor into Phospholipid Vesicles†

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ABSTRACT: Purified human placental insulin receptors were incorporated into small unilamellar phospholipid vesicles by the addition of *n*-octyl β-glucopyranoside solubilized phospholipids, followed by removal of the detergent on a Sephadex G-50 gel filtration column and extensive dialysis. The vesicles have an average diameter of 142 ± 24 nm by Sephacryl S-1000 gel filtration chromatography and 119 ± 20 nm by transmission electron microscopy. These vesicles are impermeant to small molecules as indicated by their ability to retain [γ-^{32}P]ATP, which could be released by the addition of 0.05% Triton X-100. Detergent permeabilization or freeze–thawing of the insulin receptor containing vesicles in the presence of ^{125}I-insulin indicated that approximately 75% of the insulin binding sites were oriented right side out (extravesicularly). Sucrose gradient centrifugation of insulin receptors incorporated at various protein to phospholipid mole ratios demonstrated that the insulin receptors were inserted into the phospholipid bilayer structure in a concentration-dependent manner. Addition of [γ-^{32}P]ATP to the insulin receptor containing vesicles was relatively ineffective in promoting the autophosphorylation of the β subunit in the absence or presence of insulin. Permeabilization of the vesicles with low detergent concentrations, however, stimulated the β-subunit autophosphorylation approximately 2-fold in the absence and 10-fold in the presence of insulin. Insulin-stimulated β-subunit autophosphorylation was also observed under conditions such that 94% of those vesicles containing insulin receptors had a single receptor per vesicle, suggesting that the initial β-subunit autophosphorylating activity is intramolecular. Phospho amino acid analysis of the vesicle-incorporated insulin receptors demonstrated that the basal and insulin-stimulated β-subunit autophosphorylation occurs exclusively on tyrosine residues. It is concluded that when purified insulin receptors are incorporated into a phospholipid bilayer, they insert into the vesicles primarily in the same orientation as occurs in the plasma membrane of intact cells and retain insulin binding as well as insulin-stimulated β-subunit autophosphorylating activities.

The insulin receptor is generally envisaged as an integral membrane glycoprotein composed of two M_r 135 000 (α) and two M_r 95 000 (β) subunits, linked by disulfide bonds into an M_r 350 000 heterotetrameric complex (Hedo et al., 1981; Van Obberghen et al., 1981; Massagué et al., 1981; Fujita-Yamaguchi, 1984; Boyle et al., 1985). Photoaffinity-labeling (Yip et al., 1978, 1980; Yeung et al., 1980) and affinity cross-linking studies (Jacobs et al., 1980; Pilch & Czech, 1980) have suggested that the α subunit contains the insulin binding site. Insulin binding stimulates the phosphorylation of the β subunit in intact cells, detergent soluble, and purified preparations of

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