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Evidence for Tertiary Structure in Aqueous Solutions of Human β-Endorphin As Shown by Difference Absorption Spectroscopy†

Thomas A. Bewley* and Choh Hao Li

ABSTRACT: The presence of a distinct tertiary structure in aqueous solutions of human β-endorphin has been demonstrated by difference absorption spectroscopy of thermolysin digests of the hormone and synthetic analogues. The results demonstrate that the α-amino group of Tyr[1], Lys[28], and some residue(s) between Thr[6] and Ser[10] are involved in forming and stabilizing the folded form of the molecule. Although a peptide corresponding to the first nine residues of human β-endorphin shows definite evidence of tertiary structure, the pentapeptide methionine-enkephalin does not.

The enkephalins (EK)[1] and endorphins (EP) are important, naturally occurring opioid peptides of animal origin. EK's are pentapeptides containing an NH_2-terminal tyrosine (Hughes et al., 1975). The EP's are larger and contain an EK sequence for the first five residues from the NH_2 terminus (Li, 1981). CD studies have suggested that conformation may play some role in the biological activities of EP (Li, 1981).

Although a considerable amount of α-helical structure has been reported for EP in various nonpolar solvents (Li, 1981; Yang et al., 1977; Hollősi et al., 1977; Jibson & Li, 1981; Wu et al., 1979), NMR and CD studies did not find significant

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[1] Abbreviations: EK, enkephalin; EP, endorphin; β_h-EP, human β-endorphin; β_c-EP, camel β-endorphin; CD, circular dichroism; NMR, nuclear magnetic resonance; UV, ultraviolet; ϵ_M, molar extinction coefficient; $\Delta\epsilon_M$, change in molar extinction coefficient of a difference spectral maximum; $A_{cm}^{0.1\%}$, absorptivity of a 1.0 mg/mL solution through an optical path of 1.0 cm; E/S, enzyme to substrate ratio; HPLC, high-performance liquid chromatography; SEM, standard error of the mean. The usual three letter codes for the amino acids are used, with superscripts indicating positions within the EP sequence.

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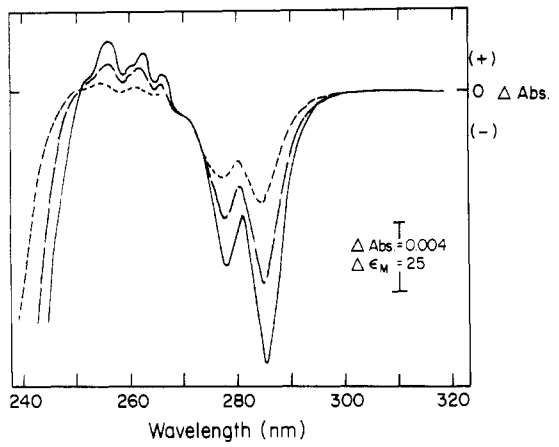

FIGURE 1: Difference absorption spectra taken at 5 (---), 20 (--), and 240 min (—), after beginning a thermolysin digestion of β_h-EP. Protein concentration 1.70×10^{-4} M (0.56 mg/mL), E/S = 1/4000. Signal to noise ratios: 20/1 at 285 nm, 15/1 at 278 nm, and 4/1 at 259 nm.

amounts of secondary or tertiary structures in aqueous solution (Yang et al., 1977; Hollősi et al., 1977; Bleich et al., 1976). A recent NMR study has shown the presence of nonrandom conformations for the Tyr[1] and Phe[4] side chains in aqueous solutions of EP (Cabassi & Zetta, 1982).

Direct evidence for the presence of a tertiary structure in aqueous solutions of β_c-EP-(1–31) has been recently reported (Nicolas et al., 1981). We have expanded these studies to include synthetic β_h-EP and 12 synthetic analogues of the human EP sequence. The presence of a tertiary structure has been confirmed, and two plausible mechanisms whereby this structure might produce specific alterations in the UV-absorption spectra of EP are presented.

Materials and Methods

All synthetic procedures, chemical characterizations, and biological properties of β_h-EP and the various analogues used have been described (Li, 1981). UV-absorption spectra were recorded at 25 °C in quartz microcells (0.5 mL capacity, 1.0 cm path) on a Perkin-Elmer, microprocessor-controlled, Model 552 spectrophotometer equipped with temperature control units on both sample and reference cell holders. All spectra were corrected for light scattering as described elsewhere (Beaven & Holiday, 1952).

Protein concentrations were determined spectrophotometrically. For β_h-EP an ϵ_M value of 2925 M^{-1} cm^{-1} at 274.8 nm ($A_{1cm}^{0.1\%}$ = 0.884) was determined from a thermolysin digest as previously described (Bewley, 1982). For all other peptides an ϵ_M was calculated from the amino acid composition assuming ϵ_M = 1420 M^{-1} cm^{-1} at 274.8–275.0 nm for each tyrosine.

Thermolysin digests were carried out at 25 °C in 0.1 M Tris-HCl buffer, pH 6.83, with an E/S ratio (w/w) varying from 1/500 to 1/5000. Usually three or more separate digestions were run on each analogue. All samples were centrifuged at 16000*g* for 10 min before use. The digests were followed by difference spectra as previously described (Nicolas et al., 1981). During some digestions, aliquots were removed at various times and submitted to HPLC by using an Alltech Vydac C-6000 (C_{18}, 300-Å porosity) column with a 0 → 30% linear gradient of 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min. The absorbance at 210 nm was recorded on a Heathkit Model SR-255-B (Benton Harbor, MI) recorder. Lyophilized HPLC peaks were hydrolyzed for 22, or 72, h in constant boiling HCl and submitted to amino acid analysis (Beckman Model 119-C automated amino acid analyzer).

Table I: Thermolysin Digestions[a] of β_h-EP-(1–31)

E/S		$\Delta\epsilon_M$[b]	$t_{1/2}$[c]
w/w	mol/mol	(M^{-1} cm^{-1})	(min)
1/500	1/5250	−94	<1
1/1000	1/10500	−95	2
1/4000	1/42000	−97	10
1/5000	1/52500	−95	15

[a] Protein concentration ≃0.3–0.4 mg/mL, 25 °C, pH 6.83. [b] Major Tyr difference peak at 285.0 nm. [c] Half-time for development of $\Delta\epsilon_M$.

Results

Figure 1 presents a typical family of difference spectra obtained during thermolysin digestion of β_h-EP. Two negative blue-shifted peaks appear at 285.0 and 278.2 nm, along with several weak difference absorption bands between 267 and 245 nm. A continuous trace of the difference absorption at 285 nm (data not shown) shows pseudo-first-order kinetics ($t_{1/2}$ ≃ 2 min for E/S = 1/1000) similar to previous data (Nicolas et al., 1981) from thermolysin digests of β_c-EP.

Table I lists the $\Delta\epsilon_M$ values for several thermolysin digestions of β_h-EP obtained over a 10-fold range in enzyme concentration. The values are seen to be independent of E/S and provide a mean ± SEM ($\Delta\epsilon_M$ = −95 ± 3 M^{-1} cm^{-1}) for the human hormone. We estimate that all of the $\Delta\epsilon_M$'s reported herein contain an uncertainty of ±3–4 M^{-1} cm^{-1}. All analogues tested gave difference spectra that were essentially identical with Figure 1, differing only in their final $\Delta\epsilon_M$ values (Figure 3).

The $\Delta\epsilon_M$ = −63 M^{-1} cm^{-1}, obtained in the present study for β_c-EP, is in reasonable agreement with previous work (Nicolas et al., 1981). Thermolysin digestion (E/S = 1/1000) of Met-EK produced no measurable difference spectrum after 6 h of incubation, also in agreement with the previous report (Nicolas et al., 1981). Direct amino acid analysis of the Met-EK digest showed no detectable free Met, or other amino acid, after a 3-h digestion.

Figure 2 presents HPLC elution patterns obtained on aliquots removed at various times during the digestion of β_h-EP. In most cases the peptide under each peak could be identified from amino acid composition. In addition, the elution positions of intact β_h-EP, β_h-EP-(1–21), and β_h-EP-(1–17) were confirmed from HPLC runs of authentic, undigested samples. Several peaks could not be unequivocally identified and presumably consist of unresolved peptide mixtures. It is evident that within the first minute, β_h-EP is cleaved to a measurable extent between residues 4–5, 16–17, 17–18, 21–22, and 25–26. It can also be seen from the relative peak heights that it is the amide bond between Phe[4] and Met[5] that is most readily cleaved. This differs from the previous report (Nicolas et al., 1981) in which the bond between Ala[21] and Ile[22] was identified as the initial site of attack. The HPLC pattern of a 1-min digest of β_c-EP-(1–31) [25 °C, pH 6.83; E/S = 1/1000] was analogous to the patterns in Figure 2, with only small shifts in the elution times of those peptides containing the slightly altered sequence between residues 27 and 31 (data not shown). This pattern indicates that in the camel sequence, it is also the bond between Phe[4] and Met[5] that is most rapidly hydrolyzed.

Discussion

Denaturation of most globular proteins produces significant

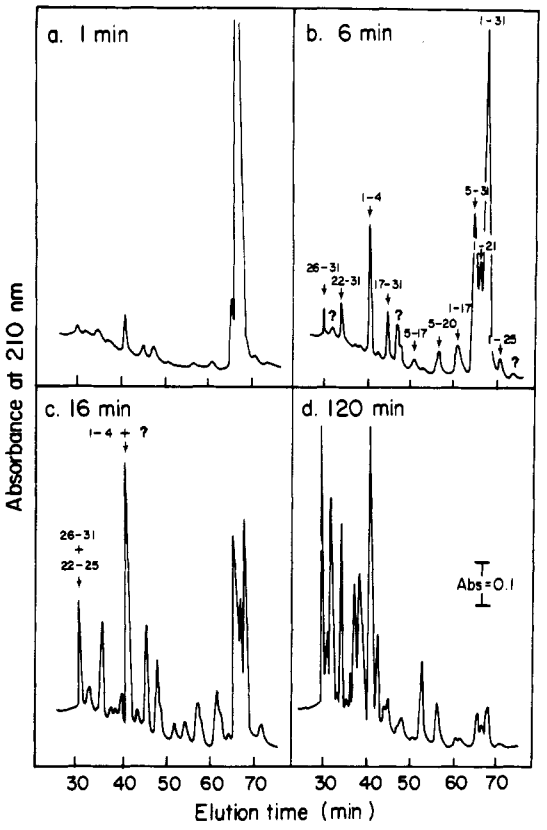

FIGURE 2: Elution profiles from reverse phase HPLC of thermolysin digests (E/S = 1/1000) of β_h-EP at 1, 6, 16, and 120 min. The peptides were eluted with a gradient of 2-propanol [0–30% (v/v) over 1 h].

alterations in the protein's UV-absorption spectrum (Beaven & Holiday, 1952; Bewley, 1982; Donovan, 1969; Wetlaufer, 1962). These denaturation effects represent that portion of the absorptivity which is contributed solely by conformational perturbation of the various chromophoric groups. In globular proteins such absorption changes are largely attributable to decreases in local refractive index around the chromophoric side chains produced by transfer from the hydrophobic interior of the folded protein out to the relatively low refractive index solvent in the denatured form (Beaven & Holiday, 1952; Donovan, 1969; Wetlaufer, 1962).

In addition, for any peptide or protein, the appearance of new, charged NH_2- and COOH-terminal groups, produced by proteolysis near chromophoric residues, can also bring about alterations in absorptivity beyond those produced by refractive index changes (Beaven & Holiday, 1952; Bewley, 1982; Donovan, 1969; Wetlaufer, 1962; Edelhoch, 1967; Herskovits & Sorensen, 1968).

Recently, a method has been devised that utilizes thermolysin digestion for "normalization" of the protein's UV absorption (Bewley, 1982). It might be expected that for smaller peptides like the EK's and β-EP's, the absorption spectrum would *already* be normalized and proteolysis would produce only those changes resulting from new charge effects. Thus, very small, net blue shifts ($\Delta\epsilon_M \simeq -3$ to -5 M^{-1} cm^{-1}) in the Phe absorption can be predicted to accompany thermolysin cleavage of Gly[3]–Phe[4], Phe[4]–Met[5], and Leu[17]–Phe[18] in β_c-EP. However, thermolysin cleavage of β_c-EP was found to produce a very significant change ($\Delta\epsilon_M = -66$ M^{-1} cm^{-1}) in Tyr absorption (Nicolas et al., 1981).

In order to investigate the nature of this phenomenon, a number of thermolysin digestions have been carried out on β_h-EP as well as various deletion analogues of the human sequence. Very low E/S ratios were employed to slow the digestions to convenient rates and to restrict optical contributions from the added enzyme to insignificant levels. A pH of 6.8–6.9 was chosen to depress any measurable ionization of Tyr phenolic groups.

$\Delta\epsilon_M$ and Fragmentation of β_h-EP. Thermolysin digestion of β_h-EP produces moderately intense blue-shifted peaks at 285.0 and 278.2 nm which may be assigned to perturbations of Tyr ring absorption (Beaven & Holiday, 1952; Donovan, 1969; Wetlaufer, 1962). Weak blue-shifted peaks, characteristic of pertubations of Phe ring absorption (Herskovits & Sorensen, 1968), also appear at 265.5, 260.5 (shoulder), 259.5, and 253–254 nm. The final intensity ($\Delta\epsilon_M = -95 \pm$ M^{-1} cm^{-1}) of the major Tyr difference peak is ≃45% greater than previously reported for β_c-EP and is independent of the E/S ratio over a 10-fold range in enzyme concentration (see Table I). This latter observation indicates that the optical effect is not simply a result of binding of the hormone (or its cleavage products) to the enzyme.

Specificity studies (Matsubara, 1970) indicate that thermolysin could cleave β_h-EP at the sites shown in Figure 3. It must be noted, however, that significant cleavage on the NH_2-terminal side of Tyr[27], though possible, is extremely unlikely. Moreover, the appearance of a free amino group on Ala[26], a cleavage known to occur extensively within the first minutes of digestion (see Figure 2), will *completely* block any subsequent hydrolysis between Ala[26] and Tyr[27] (Matsubara, 1970). As discussed below, this point is crucial to quantitative interpretation of the $\Delta\epsilon_M$ of β_h-EP. From HPLC analyses (Figure 2), it appears that in both β_h-EP and β_c-EP, it is the bond between Phe[4] and Met[5] that is most readily cleaved, although peptides corresponding to cleavages at residues 17, 18, 21, 22, and 26 also appear at the earliest times tested.

$\Delta\epsilon_M$ of Deletion Analogues. As progressively longer segments of the β_h-EP sequence are added to Met-EK, thermolysin cleavage produces increasingly intense tyrosyl blue-shifted spectra from 0 to -95 M^{-1} cm^{-1} (Figure 3). It is apparent that the increase in $\Delta\epsilon_M$ is not a smooth function of increasing chain length but occurs in more or less discrete steps. About one-third of the total appears between Thr[6] and Lys[9], with a small additional increase between Ser[10] and Leu[17]. The 9 residues from Phe[18] to Ala[26] produce no significant increase beyond that already present in the first 17 residues. A sudden, and relatively large, increase in $\Delta\epsilon_M$ occurs with addition of Tyr[27], and an equally sudden, but even larger, increment is seen upon addition of Lys[28]. Completion of the sequence with residues Lys[29] through Glu[31] has no further effect.

Perturbation of Tyr[27] Ring Absorption. In those analogues containing Tyr[1] through Ala[26], the entire blue shift at 285 nm obviously originates from the ring absorption of Tyr[1]. However, the presence of Tyr[27] in the more complete forms adds a second potential source for part of the total blue shift. Model compound studies predict that proteolysis on the NH_2 side of Tyr[27] should be accompanied by a significant charge perturbation ($\Delta\epsilon_M$ up to $\simeq -185$ M^{-1} cm^{-1}), while cleavage of Asn[25]–Ala[26] would produce a blue-shifted $\Delta\epsilon_M$ of only 18–25 units (Beaven & Holiday, 1952; Donovan, 1969; Wetlaufer, 1962). The actual value will depend on the degree of ionization of the new α-NH_2. This portion of the total $\Delta\epsilon_M$ must be considered an *artifact* of thermolysin treatment.

Two key analogues were chosen to evaluate this artifactual $\Delta\epsilon_M$. Neither β_h-EP-(6–31) nor β_h-EP-(21–31) contains Tyr[1] of the EP sequence, the chromophore responsible for the entire $\Delta\epsilon_M$ in all analogues up through Ala[26]. Therefore, the $\Delta\epsilon_M$ found on thermolysin treatment of these molecules must stem

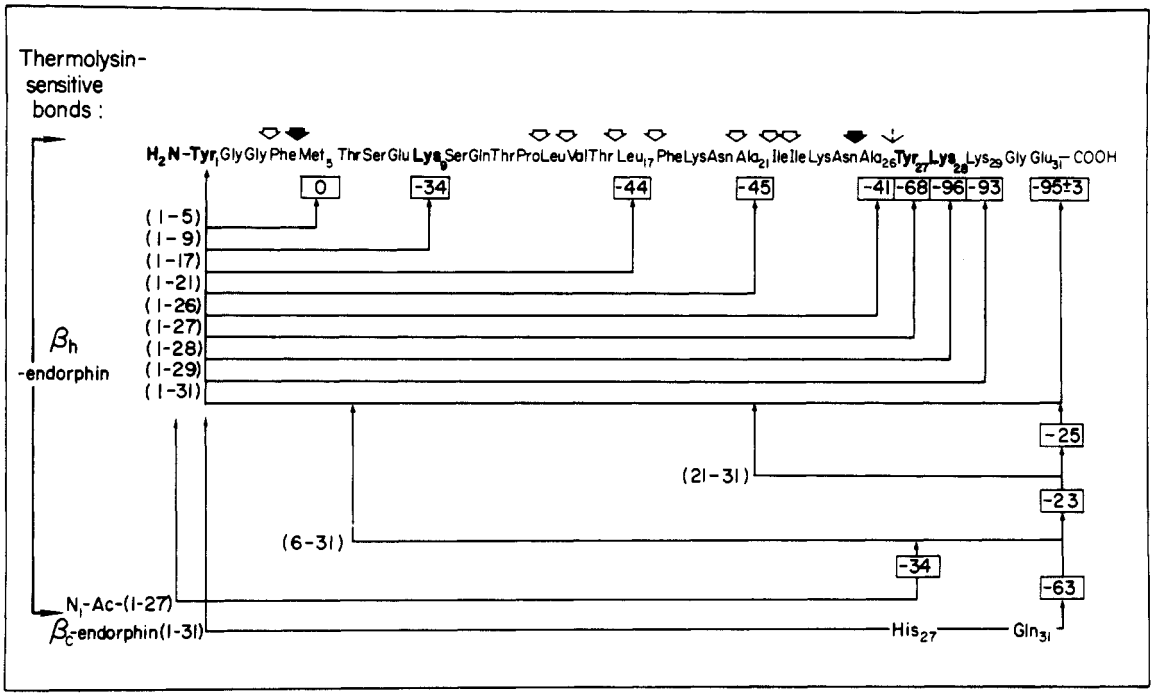

FIGURE 3: Sequence of β_h-EP showing $\Delta\epsilon_M$ values for various analogues, including β_c-EP. The positions of potential thermolysin cleavages are also shown above the sequence. Major cleavage sites thought to be responsible for the $\Delta\epsilon_M$s are shown in bold face. The dashed arrow to the left of Tyr^{27} indicates the improbability of this cleavage.

largely from the charge effect on Tyr^{27} described above. the $\Delta\epsilon_M$'s found for β_h-EP-(6–31) and β_h-EP-(21–31) are not significantly different from each other and are well within the predicted range. Furthermore, the *difference* between the $\Delta\epsilon_M$ for β_h-EP-(1–26) and that for β_h-EP-(1–27) is also very nearly this same value (i.e., $-27\ M^{-1}\ cm^{-1}$). Thus, we conclude that an artifactual blue-shifted contribution of $\simeq -25$ units is to be expected in *all* Tyr^{27}-containing analogues. It should be noted that this contribution does not indicate the presence of additional tertiary structure in these analogues. This can be further demonstrated. If the artifactual contribution is subtracted from the $\Delta\epsilon_M$ for β_h-EP, the remaining blue shift ($\Delta\epsilon_M \simeq -70\ M^{-1}\ cm^{-1}$) is reasonably close to that previously reported (Nicolas et al., 1981) and confirmed herein for β_c-EP. This argues for the presence of essentially equivalent tertiary structures in *both* EP molecules.

The substantial increase in $\Delta\epsilon_M$, seen on addition of Lys^{28}, must in large part originate from perturbation of Tyr^1 rather than Tyr^{27}. This again follows from the observation that the *nonartifactual* component of the β_h-EP $\Delta\epsilon_M$ ($\simeq -70\ M^{-1}\ cm^{-1}$) is virtually identical with the *total* value for β_c-EP ($\simeq -65\ M^{-1}\ cm^{-1}$). Thus, Lys^{28} must play the *same* tertiary structural role in both molecules, namely, perturbation of Tyr^1. Similarly, the essentially equivalent $\Delta\epsilon_M$ (composed almost entirely of the charge effect artifact on Tyr^{27}) found for β_h-EP-(6–31) and β_h-EP-(21–31) indicates that residues 6–21 do not effect the ring absorption of Tyr^{27} but probably contribute also only through interaction with Tyr^1.

The results from a third substitution–deletion analogue [Ac-Tyr^1-β_h-EP-(1–27)] can now be interpreted. This analogue contains both of the tyrosyl rings and all other residues important to the blue shift except Lys^{28}. The only other missing feature is the free α-NH_2 on Tyr^1. The $\Delta\epsilon_M$ for this molecule ($-34\ M^{-1}\ cm^{-1}$) is again almost entirely composed of the charge effect artifact on Tyr^{27} and clearly points to the crucial role played by the Tyr^1 α-NH_2 group.

Perturbation of Tyr^1 Ring Absorption. The key to understanding the tertiary structural interactions in β-EP lies in the mechanism of perturbation (red shifting) of the Tyr^1 ring absorption. Potential sources of this include hydrogen bonding of the Tyr^1 phenolic group (Strickland et al., 1972), refractive index (hydrophobic shielding) effects similar to those found in globular proteins, and charge effects on the Tyr^1 ring similar to the artifactual contribution already described for Tyr^{27}.

Hydrogen bonding of the Tyr^1 phenolic group does not provide a satisfactory explanation. There is no obvious reason why the Tyr^1 phenolic group in Ac-Tyr^1-β-EP-(1–27) should be less hydrogen bonded than that in β_h-EP-(1–27). It is also difficult to explain how this mechanism by itself can account for the apparent multistep increase in $\Delta\epsilon_M$. The available evidence points more directly to the importance of the α-NH_2 group of Tyr^1, rather than the phenolic group.

Although "burial" of the Tyr^1 ring within a hydrophobic core is difficult to envision with molecules as small as β_h-EP-(1–9), or even β_h-EP itself, refractive index effects produced by hydrophobic shielding could produce the required red shift (Wetlaufer, 1962; Donovan, 1969). However, in order to accommodate such hydrophobic shielding with the mapping results summarized in Figure 3, it is necessary to propose an additional cooperative effect. Thus, some unknown number of nonpolar side chains must be held near the Tyr^1 ring by specific conformational restraints originating from secondary interactions (hydrogen bonding and/or salt-bridge formation) involving at least Lys^{28}, the α-NH_2 group of Tyr^1, and some residues) between Thr^6 and Lys^9. The cooperative nature of this interaction is further demonstrated by the *N*-Ac-β_h-EP-(1–27) and β_h-EP-(6–31) analogues, which indicate that no significant perturbation, by any proposed mechanism, can occur without the *simultaneous* presence of the Tyr^1 α-NH_2 and either Lys^{28} or the region between Thr^6 and Lys^9. Residues Ser^{10} through Ala^{26}, which contain the highest proportion of hydrophobic side chains, produced only a small effect ($\simeq -10\ M^{-1}\ cm^{-1}$), and again only in the presence of the Tyr^1 α-NH_2. Thus, the mere presence of nonpolar side chains is not sufficient by itself to produce more than a fraction of the

total perturbation. Nevertheless, hydrophobic shielding, in concert with the type of specific restraints mentioned above, is a potentially effective mechanism. Unfortunately, a detailed model, consistant with this speculative mechanism, cannot be produced until more definite information concerning the interactions of the various NH_2 groups and identification of the relevant nonpolar side chains can be obtained.

The titratable positive charge on certain primary NH_2 groups might also be used to develop a second, quite different speculative mechanism which could also explain the red-shifted perturbation. The Tyr^1 ring absorption is very sensitive to the degree of ionization of its α-NH_2 group (Wetlaufer, 1962; Donovan, 1969). Increasing proximity of a more basic proton will favor expulsion of the α-NH_2 proton, depressing its pK_a. This partial deprotonation will, in turn, result in a red shift in the Tyr^1 ring absorption by an amount consistent with the local pH and the degree of change in pK_a. Complete deprotonation would produce a red shift at 285 nm of $\simeq 185$ M^{-1} cm^{-1}. Simple Henderson–Hasselbalch calculations suggest that at pH 6.9, a modest pK shift of −0.6 pH unit would provide the entire, nonartifactual optical effect seen for β-EP. These considerations, along with our mapping results (Figure 3), suggest the following hypothetical folding pattern and charge effect mechanism. As mentioned above, the perturbing effect of both Lys^{28} and the important region from Thr^6 through Lys^9 requires the presence of the Tyr^1 α-NH_2 group. We may envision a conformation which places all three of these elements into some degree of close proximity. The resulting concentration of positive charges (the α-NH_2 of Tyr^1, the ϵ-NH_2 of Lys^{28}, and possibly even the ϵ-NH_2 of Lys^9) will favor partial expulsion of the α-NH_2 proton, lowering the group's pK_a. The energy necessary to hold such a tertiary structure together against electrostatic repulsion might be provided both by hydrogen bonds and/or salt bridges involving neighboring residues and by the lowered α-NH_2 pK_a itself, which would act to partially neutralize the charge concentration. The α-COOH of Glu^8 is an especially attractive condidate for a potential salt bridge. This general mechanism would also allow for part of the total optical effect to still be provided by hydrophobic shielding, in addition to the charge effect. Thermolysin cleavage would destabilize all secondary interactions, leading to a loss of shielding, renormalization of the α-NH_2 pK_a, and the accompanying blue shift in Tyr^1 absorption. The immediate advantage of this mechanism is that it suggests a testable physicochemical property: the α-NH_2 pK_a. On this value alone, the charge effect model will either stand or fall. If future studies fail to demonstrate abnormal pK_a values for appropriate analogues, then the case for a purely hydrophobic shielding mechanism will be greatly strengthened. It may even be possible to ultimately partition the total $\Delta\epsilon_M$ into hydrophobic shielding and charge effect portions.

Finally, we wish to point out the efficacy of this general type of procedure for evaluating the conformational properties of small peptides or proteins where more classical methods give little information.

Acknowledgments

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Registry No. β_h-EP, 61214-51-5; β_h-EP-(1–5), 58569-55-4; β_h-EP-(1–9), 59481-79-7; β_h-EP-(1–17), 60893-02-9; β_h-EP-(1–21), 85251-32-7; β_h-EP-(1–26), 85251-33-8; β_h-EP-(1–27), 76622-84-9; β_h-EP-(1–28), 68078-30-8; β_h-EP-(1–29), 68078-31-9; β_h-EP-(21–31), 85251-34-9; β_h-EP-(6–31), 77761-27-4; *N*-Ac-β_h-EP-(1–27), 85251-35-0; β_c-EP, 59887-17-1.

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