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Proton NMR Studies of the Biologically Active 1-34 Fragment of Bovine Parathyroid Hormone: Examination of a Structural Model

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Proton NMR spectra of the biologically active 1-34 fragment of bovine parathyroid hormone (bPTH) were studied as a function of pH over the range of pH 4 to 10, in buffer and in 6 M guanidine DCl. One of the histidine C-2 peaks titrated normally, with a pK_a value of 6.8, but the other two histidines in this peptide had pK_a values of 6.3. Denatured PTH showed only one histidine C-2 peak with a pK_a of 6.7. An aliphatic peak identified as due to either a methionine or a glutamine residue also shifted with pH, and the pK_a for this shift was 6.3. Finally, small but significant upfield shifts in the methyl and methylene resonances were observed as a function of pH, and when compared to the denatured peptide. These results indicate that the N-terminal domain of native PTH has considerable structure in solution, and are consistent with a theoretical model for the folding of this peptide (10).

Parathyroid hormone (PTH)[2] is the peptide hormone primarily responsible for regulation of blood calcium. Native PTH is an 84-amino acid, single chain polypeptide. However, the biological functions of this hormone are retained by an amino-terminal fragment containing only the first 34 amino acids (1, 2). Thus, 1-34 PTH must assume much of the structure required for activation of PTH-responsive systems.

PTH has not been crystallized, and little is known about its conformation in solution. An earlier NMR study of a synthetic pentapeptide representing the 20-24 region of PTH indicated that this peptide takes on a unique structure wherein the side chain of Val 21 interacts closely with the side chain of Trp 23 (3, 4). Although it was suggested that the remainder of the 1-34 PTH fragment may exist as a random coil, there is considerable evidence from optical (5, 6), immunological (7), dark field electron microscopy (8), and chemical reactivity studies (9) indicating that the 1-34 PTH fragment does have secondary and tertiary structure in solution.

From both experimental and theoretical considerations, we proposed a model for the folding of PTH (10). This model (shown in Fig. 1) led to predictions regarding the molecular environment of specific amino acids in the N-terminal domain of PTH which are amenable to experimental examination. One such prediction is that methionine 8 is less accessible to solvent than methionine 18. This suggestion was borne out in studies of the reactivity of the individual methionine residues with hydrogen peroxide (9). A similar prediction was made with respect to the histidine residues. The model (Fig. 1) suggests that of the three histidines within the amino terminal domain, His-9 and His-32 are more likely to be folded into the interior than is His-14. To test this prediction, and to gain more information on the tertiary structure of the active domain of PTH, we have conducted proton NMR studies of 1-34 PTH.

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[2] Abbreviations used: PTH, parathyroid hormone; bPTH, bovine parathyroid hormone

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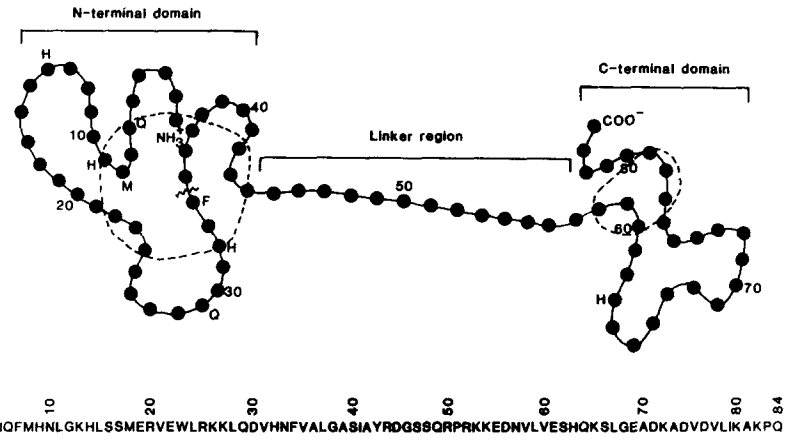

FIG. 1. Proposed folding of PTH. The 84-amino acid chain is folded into two domains separated by a linker region. The N-terminal domain is biologically active. The areas enclosed by dotted lines indicate the proposed hydrophobic core of each domain. The positions of the amino acids discussed in this paper are shown and the 1-34 peptide is delineated by the indicated break in the peptide chain. The primary structure of the hormone (bovine form) is shown below the model.

MATERIALS AND METHODS

Materials. The following reagents and solutions were used: 100 at.% D_2O, 100 at.% deuterium chloride (20% solution in D_2O), 99% sodium deuteroxide (30% solution in D_2O), Sigma guanidine hydrochloride (grade I), and reagent grade acetone. The 1-34 fragment of bovine PTH was purchased from Peninsula Laboratories, and from Bachem. The commercially provided peptide was further purified by HPLC prior to use and the biological activity was confirmed by activation of renal membrane adenylyl cyclase, using methods described earlier (11). For spectroscopy, the peptides were lyophylized out of 100 at.% D_2O three to four times, and finally dissolved in D_2O at final concentrations between 0.5 and 1 mM. A trace of acetone was added as an internal reference. The labile protons of guanidine were exchanged in D_2O by the same procedure as used with PTH.

NMR spectroscopy. ^1H NMR spectra were obtained at 270 MHz in a 5-mm probe of a Brucker WH 180/270 Fourier transform NMR spectrometer equipped with a Nicolet 1180 computer. Data were collected in the first 8192 data points of the quadrature mode, and transformed after zero filling to 32,768 data points using an exponential multiplication factor of 0.5 Hz. The 90° pulse time was 5 ms, with a total delay time of 2.000 s.

Control scans were taken of concentrated solutions of the phosphate buffer and the guanidine solutions to check for peaks due to impurities. Some impurities were found which interfered to varying extents with interpretation of the NMR spectra. One impurity which appeared as a doublet at 1.333 and 1.308 ppm did not interfere significantly and was ignored. However, 6 M guanidine had an impurity which obscured a portion of the aromatic region at basic pH. For this reason, data for certain peaks in the aromatic region could not be obtained above pH 8 when denaturing solvent was used.

Examination for aggregation of peptides. Since interpretation of the NMR data could be complicated by peptide aggregation in solution, we examined the behavior of 1-34 PTH on gel filtration columns. Solutions ranging in concentration from 5 to 0.1 mM were chromatographed on Biogel P-6 and Biogel P-30 columns. With the P-6 columns (M_r cutoff = 6 kDa) the peptide eluted slightly behind the void volume at all concentrations. With the P-30 columns the peptide eluted in a symmetrical peak of identical shape and elution position at all peptide concentrations. From these results we concluded that the peptide is a monomer at the concentrations used for the NMR studies.

Titration curve data analysis. The pH dependences of the chemical shifts were monitored for reversibility by checking chemical shifts at intermediate values after obtaining spectra at the pH extremes of a titrating residue. The data were fit to theoretical curves assuming a Hill coefficient of 1 (12, 13) by an iterative least-squares program.

RESULTS

In the theoretical model for folding of the N-terminal domain of bovine PTH (see Fig. 1) it was proposed that the aromatic residues Phe-7, His-9, Trp-23, His-32, and Phe-34 form part of a hydrophobic core.

On the other hand, His-14 was predicted to be exposed. Therefore, examination of the titration properties of the histidine residues was of primary interest. Figure 2 shows a characteristic spectrum of 1-34 bPTH in the aromatic region near neutral pH. The limited number of aromatic residues in this peptide (two Phe, one Trp, three His) made it relatively easy to obtain reasonable assignments for the major peaks in this spectrum. These assignments are indicated in Fig. 2, and were made on the basis of chemical shift values in the literature (14, 15), the relative peak areas, and the pH dependence of each residue. The histidine residues were assigned, primarily on the basis of their pH dependence (Fig. 3), to peaks A, B, C [C(2)H protons] and L, M, N [C(4)H protons]. Since each histidine gives rise to an individual resonance, it follows that all three histidines exist in different microenvironments within the 1-34 bPTH N-terminal domain.

The titration behavior of the peaks in the aromatic region is shown in Fig. 3. The data obtained for the C(2)H protons of histidine (peaks A, B, and C) clearly suggests

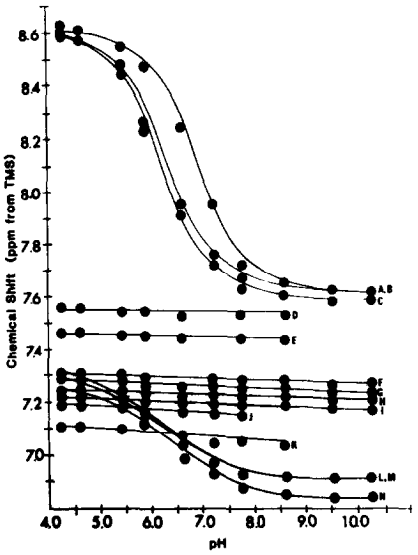

FIG. 3. Titration curve for the peaks in the aromatic region of the NMR spectrum shown in Fig. 2. The solid lines are theoretical curves constructed from the experimental data points shown. The chemical shift limits and the pK_a values for the histidine C(2)H protons appear in Table I. The titration curves for the denatured peptide are not shown, but also gave an excellent fit to the experimental points.

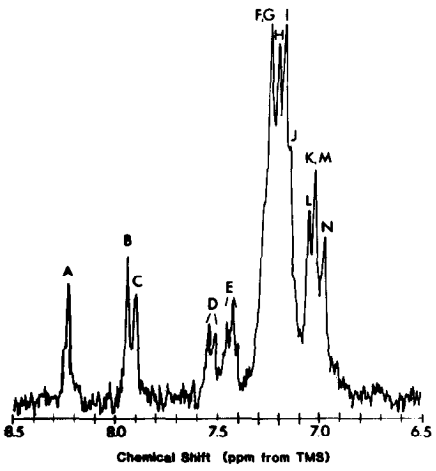

FIG. 2. The aromatic region of the ^{1}H NMR spectrum of the 1-34 fragment of bovine PTH (aromatic residues are Phe-7, His-9, His-14, Trp-23, His-32 and Phe-34). The resonances are assigned as follows: A, B, and C are histidine C(2)H; D and E are tryptophan C(7)H and C(4)H respectively; F, G, H, I, and J are phenylalanine multiplets; K is tryptophan C(5)H; L, M, and N are the histidine C(4)H resonances.

that two of the histidines are in quite similar environments, but that the third is significantly different. It was not possible to unequivocally follow the changes in the chemical shifts of the C(4)H protons, due to the extensive overlap with the Trp and Phe resonances. Thus, the reported pK values are derived only from the C(2)H protons.

As indicated in Table I, the pK_a values for the histidine residues obtained from the titration curves for the C(2)H protons are 6.3 for the two similar residues, and 6.8 for the third. The expected pK_a value for a histidine in a nonstructured environment is near 6.7 (16), suggesting that the two histidines with lower pK_a values are not fully solvent exposed. Furthermore, when 1-34 bPTH was titrated in 6 M guanidine-DCl the difference between the histidine residues was eliminated and all three residues titrated with a pK_a of 6.7 (data not shown). Thus, the differences in the histidine residues are related to the folding of the peptide chain, and two of these residues have

TABLE I

HISTIDINE TITRATION DATA FROM NMR SPECTRA OF 1-34 PTH

Peak	Proton	Chemical shift* a	Chemical shift* b	pK_a
A	C(2)H	8.598	7.626	6.8
B	C(2)H	8.597	7.629	6.3
C	C(2)H	8.600	7.586	6.3

* Chemical shifts are shown for the acid (a) form and the base (b) form.

pK values lower than expected for exposed residues. These results are consistent with our model for the folding of PTH, in the sense that two histidines are apparently in a less polar environment than a third. Thus, we suggest that the histidines with pK_a values of 6.3 are His-8 and His-32, residues that are predicted to be in a relatively nonpolar environment. Resonance A is assigned, by this reasoning, to His-14. However, clearly these assignments are not established, at the present time.

Evidence of structure was also apparent in the aliphatic region of the spectra. The most interesting data were found in the region between 2.2 and 0.6 ppm. As shown in Fig. 4, at the same pH, the spectra of the native (Fig. 4B) and denatured peptide (Fig. 4C) are significantly different in this region. Although four major peaks are seen in each spectrum, their location, fine structure and width are different in the two solvents. In addition, it is apparent from comparison of the spectra of the native peptide at pH 6.6 (Fig. 4A) and pH 10.3 (Fig. 4B) that a number of the resonances shift upfield with pH. Also, the shoulder near 0.8 ppm, formerly attributed to the CH_3 protons of Val-21 (2), is apparent in the native peptide at pH 6, but was lost in denaturing solvent (not shown). All of these effects are consistent with the suggestion that the 1-34 PTH peptide has considerable tertiary structure in solution.

The pH dependence was investigated for the aliphatic resonances identified as peaks 1-4 in Fig. 4. These data are shown in Fig. 5. The pK_a values for these shifts are 6.3 for peak 2 and 8.1 for peaks 3 and 4. The lines through the titrating peaks are theoretical curves based on these pK values. It is clear that peaks 3 and 4 move as a doublet, and based on their chemical shift, peak area, and the pK_a values, these were assigned to the methyl group of the N-terminal alanine. The pK_a values and the chemical shift limits for these resonances

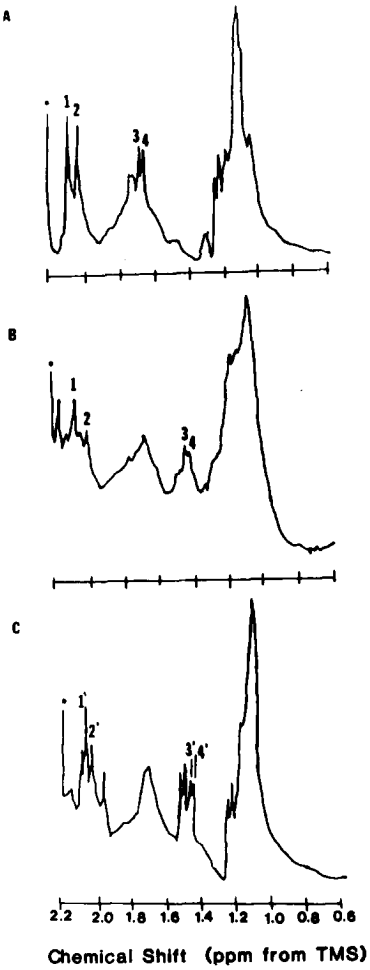

FIG. 4. Aliphatic region of the proton NMR spectrum for 1-34 PTH. (A) The native peptide spectrum at pH 6.61; (B) the native peptide at pH 10.31; (C) the spectrum of the denatured peptide at pH 10.28. The peak designated with an * in each panel is the internal reference, which is at 2.225 ppm relative to TMS. The pH dependences of the numbered resonances are shown in Fig. 5.

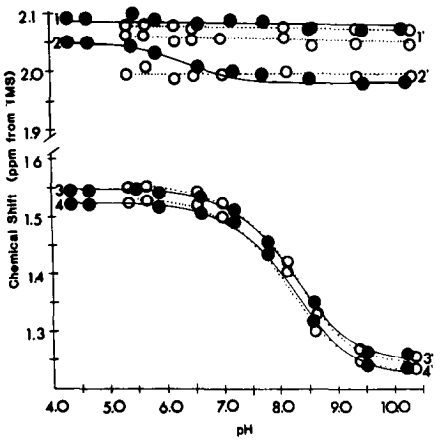

FIG. 5. Titration curves for the numbered peaks in Fig. 4. Solid circles denote the data points for the native conformation, and open circles those for the denatured peptide. Peak 2 titrates with a pK_a of 6.3 and has an area equivalent to six protons. Peaks 3, 4, and 3′, 4′ are due to the N-terminal alanine. The pK_a for the native doublet is 8.16 and for the denatured doublet, 8.21.

when the peptide was in 6 M guanidine · DCl were not significantly different from those determined for the native peptide, suggesting that this residue is freely available to solvent. This finding is not consistent with our earlier suggestion that the amino terminal residue may be buried (17).

Finally, the pH dependent shift in peak 2 (Figs. 4 and 5) is of interest. From the area of this peak we estimate that it results from five to six protons, and its chemical shift is consistent with an assignment of methionine, glutamine, glutamate, or proline. However, the peptide contains no proline residues. Interestingly, our model predicts that one methionine (Met-8), one glutamate (Glu-22), and one glutamine (Gln-6) are part of the hydrophobic core of the N-terminal domain of PTH, while one methionine (Met-18), two glutamates (Glu-4, Glu-19), and one glutamine (Gln-29) are all in exposed loops of the peptide chain. Since the pK for the shift of peak 2 corresponds to that of the two histidine residues (pK = 6.3), the data strongly suggest that the environment for this presently unidentified residue is related to histidine titration. This could result either from conformationally induced alteration in its environment, brought about by titration of the buried histidines, or from direct interaction of this residue with one or both of the low pK_a histidine residues. The fact that this peak does not titrate in the denaturing solvent supports the argument that its environment is determined by the folding of the peptide.

DISCUSSION

Earlier NMR studies of PTH centered on residues 20–24 of this peptide and the configuration of a synthetic pentapeptide containing only these residues (2, 3). Thus, the present work is the first examination of the entire NMR spectrum of the biologically active 1-34 PTH peptide and its pH dependence. The data indicate that the N-terminal domain of PTH has considerable tertiary structure in solution, and provides new information on the role of several specific residues in the formation of this structure.

The theoretical model for the structure of PTH shown in Fig. 1 is consistent with much of the NMR data. In this model, histidines 9 and 32 are near regions of the peptide with high hydrophobicity and are part of the chain predicted to fold inward, forming the hydrophobic core of this part of the PTH molecule. On the other hand, histidine 14 is predicted to be in an exposed region of the peptide chain. Since a hydrophobic environment would lead to a reduced pK_a for histidine, the fact that two of the three residues have pK_a values lower than the third, and lower than predicted for an exposed histidine, is in agreement with this suggestion. Furthermore, the observation that a methionine or a glutamine residue, or both, shifts when a histidine is titrated (Figure 5) is also consistent with our model. Earlier work (9) showed that Met-8 is less accessible to solvent than Met-18 in 1-34 PTH, as predicted by our model, and titration of either His-9 or His-32 could alter the environment of this methionine. The NMR data indicating structural changes associated with titration of histidine residues are also consistent with

earlier circular dichroism studies which show that significant changes in helix content occur between pH 4 and 7, both in the native hormone and the 1–34 fragment (5).

The titration data for the amino terminal alanine suggests that the N-terminus is probably exposed to solvent and this is not consistent with our earlier suggestion that this residue may also be part of the hydrophobic core (15). That suggestion was based on the reduced chemical reactivity of the amino terminus with acetimidate esters when compared to free alanine. It may be that those differences are due to inherent chemical differences between the free amino acid and a peptide in general, rather than to the folding of the peptide chain. The significance of the molecular environment of the N-terminal residue is not certain at the present time. The human and porcine forms of the hormone have a serine rather than an alanine at this position, and these are less potent in the renal membrane adenylyl cyclase bioassay system, suggesting that a hydrophobic residue at this position is important. On the other hand, since these natural forms of the hormone are obviously functional, this consideration is apparently not a determining factor in the ability of the peptide to assume its biologically active configuration.

In summary, the NMR data clearly support the conclusion that the 1–34 fragment of bPTH has a considerable amount of structure in solution. Much of the data are supportive of our proposed model for this structure. Further NMR studies using two-dimensional techniques will provide more detailed information on the specific interactions of various residues in this structure. The 1–34 fragment of PTH is an ideal peptide for such studies since it is small enough to allow assignment of many of the protons of interest, yet large enough to assume a folded structure in solution.

REFERENCES

1. TREGEAR, G. W., VANRIETSCHOTEN, J., GREENE, E., KEUTMAN, H. T., NIALL, H. D., REIT, B., PARSONS, J. A., AND POTTS, J. T. JR. (1973) *Endocrinology (Baltimore)* **93,** 1349–1353.
2. POTTS, J. T., JR., KRONENBERG, H. M., AND ROSENBLATT, M. (1982) *Adv. Prot. Chem.* **25,** 323–369.
3. BUNDI, A., ANDREATTA, R., RITTEL, W., AND WUTHRICH, K. (1976) *FEBS Lett.* **64,** 126–129.
4. BUNDI, A., ANDREATTA, R. H., AND WUTHRICH, K. (1978) *Eur. J. Biochem.* **91,** 201–208.
5. BREWER, H. B., FAIRWELL, T., RONAN, R., RITTEL, W., AND ARNAUD, C. D. (1975) *in* Calcium Regulating Hormones (Talmadge, R. V., Owen, M., and Parsons, J. A., Eds.), pp. 23–32, Elsevier, New York.
6. COHN, D. V., AND MACGREGOR, R. R. (1981) *Endocrine Rev.* **2,** 1–26.
7. HABENER, J. F., AND POTTS, J. T., JR. (1979) *Endocrinology (Baltimore)* **105,** 115–119.
8. FISKIN, A. M., COHN, D. V., AND PETERSON, G. S. (1977) *J. Biol. Chem.* **252,** 8261–8268.
9. FRELINGER, A. L., AND ZULL, J. E. (1985) *Arch. Biochem. Biophys.*, in press.
10. ZULL, J. E., AND LEV, N. (1980) *Proc. Natl. Acad. Sci. USA* **71,** 3791–3796.
11. ZULL, J. E., AND CHUANG, J. (1984) *Anal. Biochem.* **140,** 214–222.
12. DWEK, R. A. (1973) Nuclear Magnetic Resonance in Biochemistry, p. 107, Clarendon Press, Oxford.
13. EDWARDS, B. F. P., AND SYKES, B. D. (1981) *Biochemistry* **20,** 4193–4198.
14. JAMES, T. C. (1975) *in* NMR in Biochemistry, pp. 242–243, Academic Press, New York.
15. JARDETZKY, O., AND ROBERTS, G. C. K. (1981) *in* NMR in Molecular Biology, p. 152, Academic Press, New York.
16. COHEN, J. S. (1971) *Biochim. Biophys. Acta* **229,** 603–609.
17. ZULL, J. E., CHUANG, J., AND BOTTI, R. (1979) *Proc. Int. Cong. Biochem.* **12,** 608.