^{31}P NMR CHARACTERIZATION OF BOVINE, EMBRYONIC, DENTAL ENAMEL PHOSPHOPEPTIDES IN SOLUTION

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Received 7 May 1980

1. Introduction

Homogenous preparations of the two major phosphopeptides, E_3 and E_4 [1,2] of embryonic bovine dental enamel have been studied in solution by ^{31}P NMR. The results demonstrate that the protein-bound phosphorus exists in solution as a phosphate monoester. No evidence was obtained for the existence of either complex phosphate bonds such as a mixed anhydride with carboxyl side chain groups, or the physical shielding of the phosphomonester due to conformation or aggregation of the peptides [3].

2. Materials and methods

2.1. *Preparation of enamel proteins and the E_3 and E_4 phosphopeptides*

The neutral soluble proteins of embryonic bovine enamel (NSEP) [4], containing >95% of the proteins of enamel [5,6], and homogenous samples of E_3 and E_4 phosphopeptides were prepared as in [1,2,4]. SDS–polyacrylamide gel electrophoresis revealed single, sharp bands for each of the two peptides, and amino acid analyses confirmed their identity [1,2].

O-phosphoserine [Ser(P)], *O*-phosphothrenonine [Thr(P)] Sigma Chemical Co., St Louis) and β-casein (a gift of Dr David Waugh, MIT, Cambridge, MA) were also used in the ^{31}P NMR studies.

2.2. *^{31}P NMR spectroscopy*

The NSEP, E_3 and E_4, β-casein, Ser(P) and Thr(P) were dissolved in 6 M urea and 6 M guanidine–HCl solutions since the E_3 and E_4 enamel phosphopeptides were soluble only in those conditions. The pH of the solutions, measured before and after each ^{31}P NMR experiment, varied from 11–1, the changes being effected by the addition of NaOH or HCl. The protein and amino acid concentrations of the solutions varied from 7–25 mg/ml, respectively.

^{31}P NMR spectra were obtained using JEOL FX-60 and Nicolet wide-bore 360 spectrometers located at the Harvard Medical School, and a Brucker HX-270 spectrometer located at the Francis Bitter National Magnet Laboratory at MIT.

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3. Results

The ^{31}P NMR results can be summarized as follows:

(1) The NSEP and the E_3 and E_4 peptides dissolved in 6 M urea showed a sharp resonance which on pH titration, and when compared with Ser(P), Thr(P) and β-casein as standards, could be clearly identified as being due to the presence of phosphomonester (fig.1A,B). The values for the ^{31}P NMR resonances of Ser(P) and Thr(P) differ somewhat especially at very low pH values (fig.1A,B). The similarity of the ^{31}P NMR resonances of the NSEP and the E_3 and E_4 peptides and the amino acid Ser(P), as well as β-casein, a protein which contains essentially only Ser(P) as its phosphorylated amino acid, are consistent with analytical chemical data that show Ser(P) but no Thr(P) in the enamel proteins [7].

(2) The shift with the pH of the resonance of the phosphomonester group in Ser(P), Thr(P), β-casein, NSEP and E_3 and E_4 phosphopeptides dissolved in 6 M guanidine–HCl were essentially identical, but all differed significantly from the

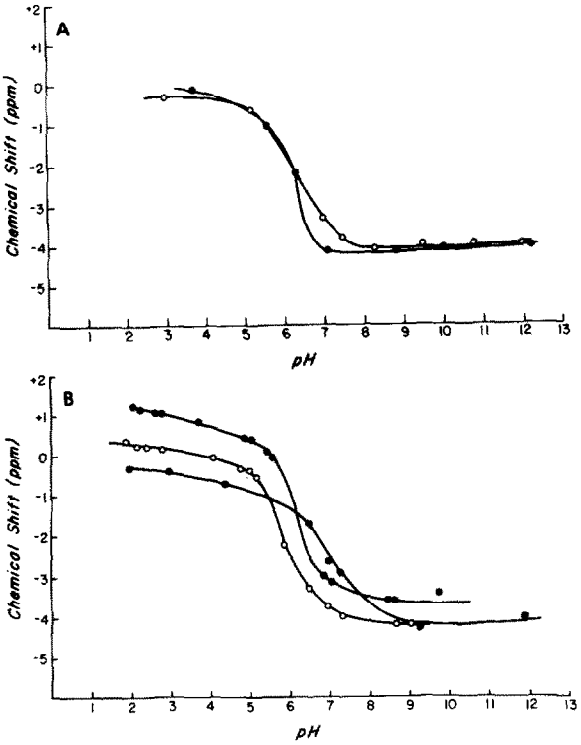

Fig.1. (A). Variation with pH of the ^{31}P NMR chemical shifts (expressed relative to 85% H_3PO_4) of the NSEP (●——●) and of the E_4 peptide (○——○) in 6 M urea. A similar curve was obtained from the E_3 peptide. (B) Similar data obtained from β-casein (●——●) and for the phosphoamino acids Ser(P) (○——○) and Thr(P) (⊖——⊖).

pH dependency curve of the NMR resonances generated when these compounds were dissolved in 6 M urea (fig.2A–D).

4. Discussion

As pointed out by Taborsky [8], analytical chemical analyses to identify specific phosphorylated amino acids in a protein may be seriously misleading. In the first place, the hydrolysis conditions used to liberate the phosphoamino acids may either destroy labile organic phosphates or produce intramolecular migrations. In addition, complex mixed bonds such as those between organic phosphorus and carboxylic acid groups may also be cleaved [9]. ^{31}P NMR of the intact phosphoprotein either avoids these difficulties, or if cleavage or intramolecular migration does occur, is capable of detecting them. In the present instance,

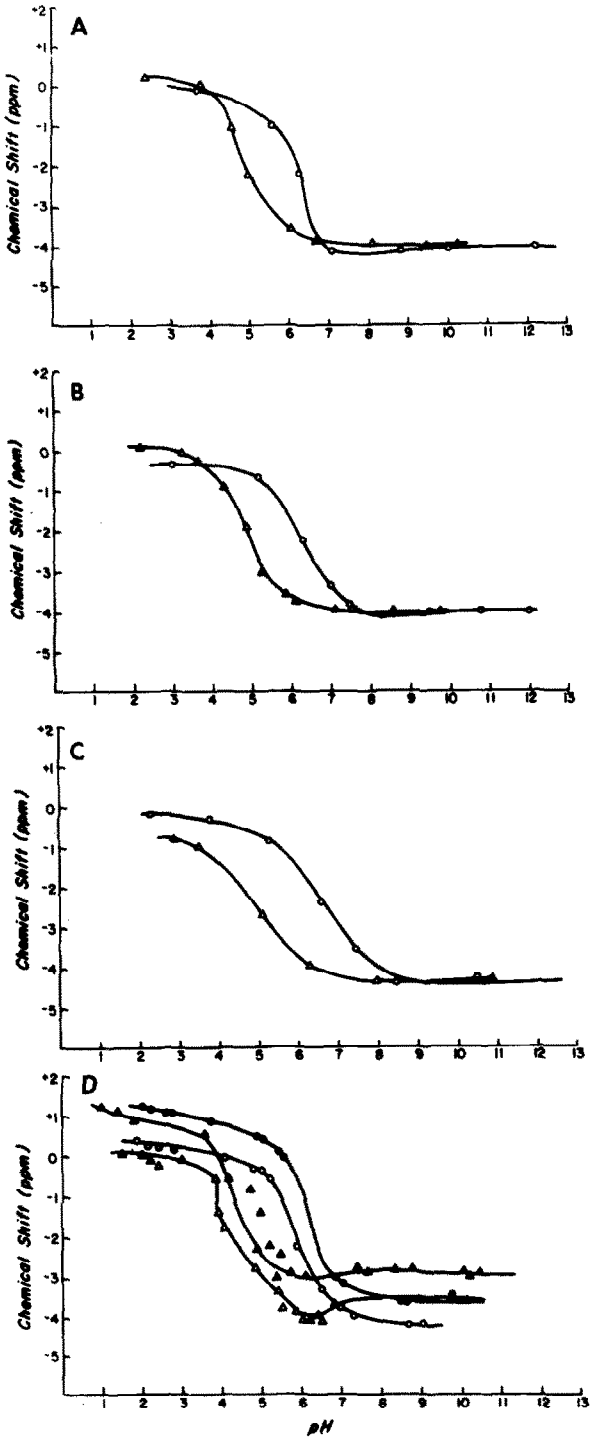

Fig.2. Variation with pH of the ^{31}P NMR chemical shifts (expressed relative to 85% H_3PO_4) from the organic phosphorus resonance in 6 M urea (circles) and 6 M guanidine–HCl (triangles) of: (A) NSEP; (B) E_4 peptide (similar results obtained from the E_3 peptide); (C) β-casein; (D) Ser(P) (○——○ and ▲——▲) and Thr(P) (●——● and ▲——▲).

the ^{31}P NMR data are clearly consistent with those obtained by analytical chemical analyses, namely that the organic phosphorus moiety of the phosphopeptides of embryonic bovine enamel are present as phosphomonesters, more specifically as Ser(P). Moreover, when the peptides are in solution there is no evidence by ^{31}P NMR for the presence of complex phosphate bonds [9] or the shielding of phosphate groups from the solvent even when the phosphopeptides were examined as components of the NSEP, that is, in the presence of the many non-phosphorylated protein components of the enamel under which circumstances a great deal of interaction and aggregation between components occur [10–12]. Similar results have been obtained from the dentinal phosphoproteins [13].

The suggestion that in the E_3 and E_4 peptides some of the phosphomonester groups were shielded or in hydrophobic regions of the peptides or peptide-aggregates [3], based on the finding that the phosphate groups did not protonate above pH 6.5 when dissolved in 6 M guanidine HCl, has been shown in the present study to be the result of an interaction between guanidine–HCl and the phosphomonester, since similar results were obtained for the phosphomonester groups in the amino acids Ser(P) and Thr(P) and the phosphoprotein, β-casein. The data of course do not preclude the possibility that other labile, phosphorylated amino acids and/or complex phosphate bonds exist in the phosphoproteins when they are in the solid state as part of the organic matrix of enamel in vivo. To examine these possibilities, studies of the enamel proteins in the solid state by ^{31}P NMR using the 'magic angle' technique [14,15] are now underway.

Acknowledgements

This paper was supported in part by grants from the National Institutes of Health (AM 15671), the American Heart Association Grant-in-Aid (76-900), the Liberty Mutual Insurance Company and the New England Peabody Home for Crippled Children, Inc. S. L. was supported by the NIH Training Grant AM 07112 to the Department of Orthopaedic Surgery, Harvard Medical School.

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