Studies of the Secondary Structures of Amelogenin from Bovine Tooth Enamel†

V. Renugopalakrishnan,‡ E. S. Strawich,‡ P. M. Horowitz,§ and M. J. Glimcher*,‡

Laboratory for the Study of Skeletal Disorders and Rehabilitation, Department of Orthopedic Surgery, Harvard Medical School, Children's Hospital, Boston, Massachusetts 02115, and Department of Biochemistry, The University of Texas Health Sciences Center, San Antonio, Texas 78284

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ABSTRACT: Circular dichroism and Fourier transform infrared spectroscopic studies of the major amelogenin protein of developing bovine tooth enamel in solution and in the solid state suggest a unique secondary structure containing β-sheet and repetitive β-turn structures. The repetitive β-turn structure at the C-terminal end results from the unique primary structure of amelogenin.

The principal proteins of developing enamel, characterized by their high concentrations of proline, glutamic acid, leucine, and histidine (Glimcher & Levine, 1966; Eastoe, 1979; Eggert et al., 1973), have been termed the "amelogenins" (Eastoe, 1979). Recent physicochemical studies (Strawich et al., 1985) and in vivo radiolabeling of the proteins (Strawich & Glimcher, 1985) have confirmed earlier studies (Eggert et al., 1973) that the major, highest molecular weight amelogenin, the putative extracellular precursor of many of the lower molecular weight components, is a protein of $M_r \sim 18\,000$. This corresponds well with the molecular weight calculated from the amino acid sequence (Takagi et al., 1984). Earlier X-ray diffraction data of the ethylenediaminetetraacetic acid (EDTA)-insoluble organic matrix of developing bovine enamel (Glimcher et al., 1961), which constitutes over 95% of the total protein content of the tissue, and of the major amelogenin fractions (Glimcher et al., 1964a,b), as well as optical rotatory dispersion studies in solution of the amelogenins (Bonar et al., 1984), showed clear evidence of a cross β-configuration in the solid state and of a β-configuration in solution. More recently, electron diffraction of the developing organic matrix of rat enamel also demonstrated the presence of a component with a β-configuration (Traub et al., 1985).

In the present study, circular dichroism (CD) and Fourier transform infrared spectroscopy (FT-IR) have been utilized to investigate the secondary structure of the major, highest molecular weight, extracellular amelogenin of developing bovine enamel (Strawich et al., 1985) in solution and in the solid state. The detailed experimental studies suggest a mixed β-turn/β-sheet model with a low α-helical content.

The occurrence of a large number of Pro residues (~300 residues per 1000 residues) and β-turn promoting residues [i.e., Asn, Gly, His, and Gln (Chou & Fasman, 1978)] in the major amelogenin (Strawich et al., 1985) strategically inserted proximally to the Pro residues [see Takagi et al. (1984) for amino acid sequence] can be expected to result in a series of contiguous β-turns at the C-terminal end. The secondary structure also contains many β-sheet subdomains which coexist with the numerous β-turns.

Materials and Methods

The major, highest molecular weight bovine amelogenin was prepared from the enamel of developing, premolar teeth as previously described (Strawich et al., 1985). In this specific instance, the major amino acids in this component were proline (310 residues/1000), glutamic acid (195 residues/1000), leucine (95 residues/1000), and histidine (82 residues/1000).

CD and FT-IR Spectroscopic Studies in Solution. (A) CD Spectroscopic Studies. A JASCO 500C spectropolarimeter was used to obtain the CD spectra of bovine amelogenin in solution. The CD spectra of bovine amelogenin in tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.2) and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) buffer whose pH was adjusted to 1.6 by the addition of microliter amounts of 1 M HCl, at concentrations of 0.5 mg/mL, were measured at room temperature by using a 0.01-cm path-length cell. A mean residue molecular weight of 115 was assumed in the calculation of molar ellipticity, θ.

(B) FT-IR Spectroscopic Studies. FT-IR spectra of bovine amelogenin solutions were obtained by using a Digilab FTS-14 FT-IR spectrometer. The attenuated total reflection (ATR) technique with a germanium crystal at 45° angle phase was utilized. The FTS-14 FT-IR spectrometer was equipped with a mercury–cadmium telluride (MCT) detector. A total of 400 scans at a resolution of 8 cm^{-1} were recorded at room temperature. The following concentrations of bovine amelogenin in different buffer solutions, covering a wide range of pHs, were used for FT-IR studies: 0.275 mg/mL (KCl–HCl buffer, pH 1.8); 0.275 mg/mL (KCl–HCl buffer, pH 2.25); 0.275 mg/mL (CH_3COOH–CH_3COONa buffer, pH 4.8); 0.175 mg/mL (piperazine dihydrochloride–NaOH buffer, pH 5.86); 0.035 mg/mL (NH_4HCO_3 buffer, pH 8.17); 0.069 mg/mL (Tris buffer, pH 9.23).

CD and FT-IR Spectroscopic Studies of Amelogenin in the Solid State. FT-IR Spectroscopic Studies. FT-IR spectra of bovine amelogenin in the solid state were recorded on a Digilab FTS-14 spectrometer at room temperature. The spectrometer was equipped with a Triglycine (deuterated) sulfate (TGS) detector. A diamond cell was used to hold the sample. A total of 400 scans at a resolution of 4 cm^{-1} were collected for each spectrum.

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* Correspondence should be addressed to this author at the Laboratory for the Study of Skeletal Disorders and Rehabilitation, Children's Hospital.

‡ Harvard Medical School.

§ The University of Texas.

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Calculation of Percentage Secondary Structures from CD Data. A program written in language C for a VAX 11/780 computer at the Harvard School of Public Health computing facility was used for the calculation of percentage secondary structures from CD data. The percentage secondary structure was calculated by using an algorithm proposed by Chang et al. (1978). The above algorithm was derived from observed CD spectra of 15 proteins with well-established secondary structures. The mean residue ellipticity, $[\theta]$, at any wavelength, λ, is expressed as

$$[\theta]_\lambda = f_{\alpha\text{-helix}}[\theta]_{\alpha\text{-helix}}{}^n + f_{\beta\text{-sheet}}[\theta]_{\beta\text{-sheet}} + f_{\beta\text{-turn}}[\theta]_{\beta\text{-turn}} + f_{\text{unordered}}[\theta]_{\text{unordered}}$$

where $[\theta]_{\alpha\text{-helix}}$, $[\theta]_{\beta\text{-sheet}}$, $[\theta]_{\beta\text{-turn}}$, and $[\theta]_{\text{unordered}}$ are the reference values for α-helical, β-sheet, β-turn, and unordered structures and f's are fractions of each of the structures, in 15 proteins whose secondary structures in solid phase are well established. A nonlinear least-square procedure is used to solve the equations. $[\theta]$ values from 190 to 240 nm at 1-nm intervals are the input to the computer program.

Conformational Assignments of Bands. The conformational assignments of amide I and III bands followed in this study are in accord with earlier studies and from data derived from numerous FT-IR studies completed in 1983–1985 in our laboratory. Because of the widespread use of FT-IR spectroscopy in protein structural analysis, the accuracy of frequencies, free from solvent interference, which have been reported has improved significantly.

(A) α-Helical Structures. The amide I and III frequencies of α-helical structures occur in the 1645–1659 cm^{-1} and 1262–1300 cm^{-1} regions, respectively. The above assignments are adapted from Parker (1983), Nevskaya and Chirgadze (1976), and the recent FT-IR photoacoustic study of poly(γ-benzylglutamate) [see Renugopalakrishnan & Bhatnagar, (1984) and references cited therein]. However, anomalously high amide I bands, i.e., 1667 cm^{-1}, have been observed in the IR spectrum of the purple membrane of *Halobacterium halobium* (Rothschild & Clark, 1979a,b) which has been attributed to the α_{II} conformation (Krimm & Dwivedi, 1982). Similarly, an anomalously low amide I frequency of 1645 cm^{-1} has also been reported for poly(lysine hydrochloride), the α'' form at 100% humidity (Blout & Lenarmont, 1957).

(B) β-Sheet Structures. We are following the assignments proposed by Chirgadze and Nevskaya (1976a,b). Antiparallel β-sheet structures manifest A and B_1 components in the 1670–1690 cm^{-1} region in IR spectra and a B_2 component around 1629 cm^{-1} (Chirgadze & Nevskaya, 1976a). The B_3 component is rarely observed. In contrast, the parallel β-sheet structures manifest a B_2 component around 1640 cm^{-1} (Chirgadze & Nevskaya, 1976b). Extensive tabulations of the literature may be found in Nevskaya and Chirgadze (1976a,b). Amide III frequencies occur in the 1230–1245 cm^{-1} region (Parker, 1983). The above studies have also been corroborated by an FT-IR study of phosvitin (Renugopalakrishnan et al., 1985b).

(C) β-Turn Structures. The amide I vibrations of β-turn structures have been a subject of detailed studies in the last 7 years. We are following the theoretical calculations of Bandekar and Krimm (1979), Lagant et al. (1984a,b), and experimental studies (Ishizaki et al., 1981; Han et al., 1980, 1981; Fox et al., 1981; Tu et al., 1978, 1979; Hruby et al., 1978; Tu, 1982; Renugopalakrishnan et al., 1985a–c; Seaton, 1983; Bhatnagar et al., 1985). Recently, it was observed from Raman studies that polypeptides containing an extensive network of β-turns manifest amide I and III bands at 1673, 1232, and 1243 cm^{-1}, respectively (G. J. Thomas, Jr., and D. W. Urry, unpublished results).

(D) Unordered Structures. By its very definition, it is quite difficult to assign a particular subregion of the amide I region to unordered structures. Nevertheless, the 1654–1657 cm^{-1} region appears to characterize unordered structure in IR spectra on the basis of IR study of feather keratin (Hsu et al., 1976) and denatured proteins [see Wallach et al. (1970), Hsu et al. (1976), and Koenig & Tabb (1980)].

(E) Mixed Domains of Extensive β-Turns and Less Extensive β-Sheet Structures. From the well-established β-sheet vibrations and the newly established β-turn vibrations, the characteristic vibrations for a protein containing mixed domains should be reasonably expected to occur between 1670–1680 and around 1240 cm^{-1}, and this assumption is amply demonstrated by the 1677 cm^{-1} amide I band which occurs in the neuromuscular blocking agent anthopleurin A (Ishizaki et al., 1979) in which NMR studies have demonstrated the coexistence of type II β-turn and antiparallel β-sheet structures.

Resolution Enhancement of FT-IR Amide I, II, and III Bands. Resolution enhancements of FT-IR bands in the amide I, II, and III regions were performed by using the algorithm developed by Kauppinen et al. (1981). In the above procedure, the inverse Fourier transform of the spectrum is multiplied by $D_g(x)/\mathcal{F}^{-1}[E_0(\bar{\nu})]$ where $1/\mathcal{F}^{-1}[E_0(\bar{\nu})] = 1/\exp(-2\pi\sigma|x|)$ results in the self-deconvolution. The line shape in the Fourier self-deconvoluted spectrum is controlled by $D_g(x)$, a general apodization function related to the resolution enhancement factor, K. A proper choice of K and σ, the half width of the Lorentzian line, is crucial for a successful deconvolution. These two parameters, whose choice is critical for a proper deconvolution of bands, are chosen by an optimization procedure to avoid artifacts (Yang & Griffiths, 1981). Its application to protein structures was recently discussed by Yang et al. (1985) and Renugopalakrishnan and Rapaka (submitted for publication).

Results

CD and Fourier Transform Infrared Spectroscopic Studies of Bovine Amelogenin in Solution. (A) CD Spectroscopic Studies in Solution. CD spectra of bovine amelogenin at pH 1.6 and 7.2 are presented in panels A and B, respectively, of Figure 1. The molar ellipticities, $[\theta]$, at pH 1.6 and 7.2 are listed in Table I.

(B) FT-IR Spectroscopic Studies in Solution. Resolution-enhanced FT-IR (difference) spectra of bovine amelogenin solution at pH 1.8 and 2.25, 4.8 and 5.86, and 8.17 and 9.23 are shown respectively in Figures 2–4. The observed frequencies and their tentative assignments are listed in Table II. The variation of the amide I and III frequencies as a function of pH is shown in Figure 5. At pH 5.86, the amide I band occurs as a doublet with frequencies of 1667 and 1632 cm^{-1}; the amide II band is a triplet with frequencies of 1580, 1551, and 1524 cm^{-1}, respectively; the amide III band occurs as a doublet with frequencies at 1281 and 1250 cm^{-1}, respectively (Figure 3B). On lowering the pH to 4.8 (Figure 3A), the amide I bands shift to 1670 and 1620 cm^{-1}, amide II collapses into a doublet with frequencies of 1547 and 1520 cm^{-1}, and amide III occurs as a triplet with frequencies at 1288, 1258, and 1239 cm^{-1}.

On further lowering the pH to 2.25 (Figure 2B), amide I shifts to 1667 and 1636 cm^{-1}, amide II becomes a quadruplet with frequencies at 1583, 1562, 1543, and 1520 cm^{-1}, and amide III retains its triplet pattern with frequencies of 1279, 1242, and 1223 cm^{-1}, respectively. At the low pH of 1.8

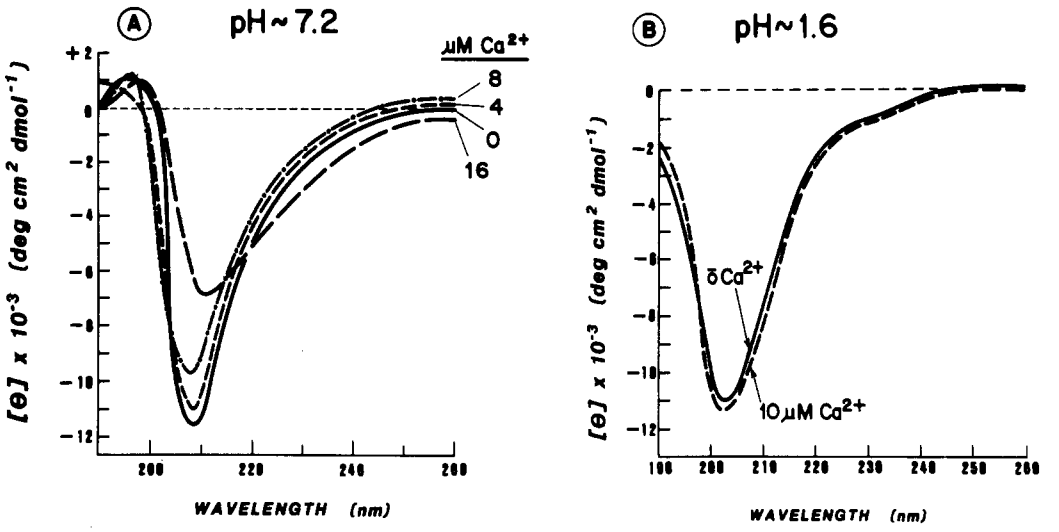

FIGURE 1: CD spectra of bovine amelogenin from 190 to 260 nm. The spectra were recorded at room temperature on a JASCO 500C spectropolarimeter controlled by a dedicated computer. Analyses of CD spectra were performed by using the procedure suggested by Chang et al. (1978). (A) In Tris buffer, pH 7.2, at a concentration of 0.5 mg/mL. Calcium was introduced as a $CaCl_2$ solution at the concentration levels indicated. (B) In Pipes buffer with the pH adjusted to 1.6 by the addition of microliter amounts of 1 M HCl at a concentration of 0.6 mg/mL. Calcium was introduced as a $CaCl_2$ solution at the concentration levels indicated.

Table I: Bovine Amelogenin CD Spectral Data and Their Conformational Analysis

pH	λ (nm)	θ (deg·cm²·dmol⁻¹)	secondary structure
7.2			
min	208	−11666	α-helix, 16%
max	196	1167	β-sheet, 30%
			β-turn, 21%
			unordered, 33%
1.6			
min	203	−11000	α-helix, 0%
			β-sheet, 58%
			β-turn, 3.5%
			unordered, 38.5%
7.2			
4 μM Ca^{2+}			
min	208	−11166	α-helix, 16.5%
			β-sheet, 30.5%
max			β-turn, 21%
			unordered, 32.5%
8 μM Ca^{2+}			
min	208	−9766	α-helix, 13.5%
			β-sheet, 38.5%
max	196	1211	β-turn, 17.5%
			unordered, 30.5%
16 μM Ca^{2+}			
min	211	−6900	α-helix, 6.5%
			β-sheet, 61%
max	199	1110	β-turns, 8.5%
			unordered, 24%

(Figure 2A), amide I continues to be a doublet with frequencies of 1667 and 1636 cm^{-1}. Amide II collapses into a doublet with frequencies of 1547 and 1520 cm^{-1}. Amide III retains its triplet structure with frequencies of 1265, 1258, and 1238 cm^{-1}, respectively.

On raising the pH to 8.17 (Figure 4A), amide I shifts to 1678 and 1651 cm^{-1}, and the amide II and III bands retain their triplet and doublet structures, respectively, with frequencies at 1582, 1559, and 1516 cm^{-1} and 1269 and 1242 cm^{-1}. At pH 9.23 (Figure 4B), the amide I band shifts to a lower frequency of 1670 cm^{-1} with a shoulder at 1640 cm^{-1} while amide II occurs as a doublet at 1551 and 1520 cm^{-1}, respectively. Amide III also occurs as a doublet at 1277 and 1246 cm^{-1}, respectively.

FT-IR Spectroscopic Studies in the Solid State. The resolution-enhanced FT-IR spectrum of solid bovine amelogenin is shown in Figure 6. Panel A of Figure 6, ranging from 1400 to 1800 cm^{-1}, shows the amide I and II regions, respectively. Panel B of Figure 6 presents the amide III region. The frequencies of the major bands and their tentative assignments are listed in Table II.

The amide I band is observed in Figure 6A as a doublet with two peaks at 1680 and 1635 cm^{-1}. The amide II band occurs at 1520 cm^{-1} with a shoulder at 1545 cm^{-1} (Figure 6A). The amide III band occurs at 1242 cm^{-1} with a shoulder at 1275 cm^{-1} (Figure 6B).

Table II: Fourier Transform Infrared Frequencies and Assignments of Major Bands of Bovine Amelogenin in Solution and in the Solid State

solution at pH							
1.8	2.25	4.8	5.86	8.17	9.23	solid	assignment
				Amide I			
1667	1667	1670	1667	1678	1670	1680	repetitive β-turn/β-sheet structure
1636 (sh)	1636	1620	1632	1651	1640 (sh)	1635	β-sheet structure
				Amide II			
1547	1583	1547	1580	1582	1551	1545 (sh)	conformational assignments not well established
	1562	1520	1551	1559	1520	1520	
1520	1543		1524	1516			
	1520						
				Amide III			
1265	1279	1288	1281	1269	1277	1275 (sh)	β-turn repetitive
1258	1242	1258	1250	1242	1246	1242	β-turn structure
1238	1223	1239					β-sheet structure

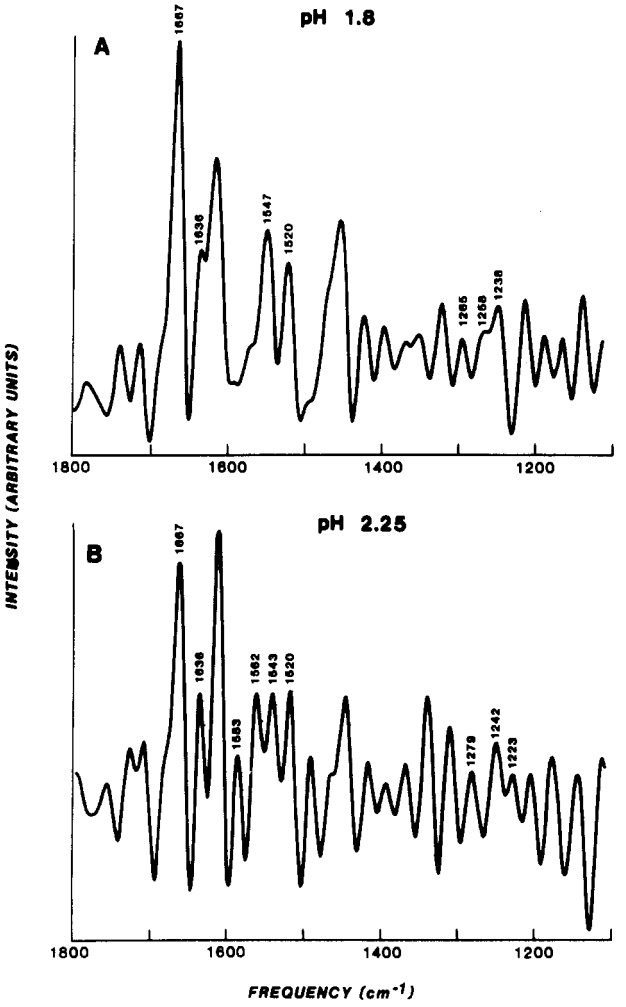

FIGURE 2: Resolution-enhanced Fourier transform infrared difference (after subtraction of buffer) spectra of bovine amelogenin in solution. An attenuated total reflectance (ATR) technique with a Ge crystal at a 45° angle phase was utilized. Spectra were recorded on a Digilab FTS-14 FT-IR spectrometer equipped with a mercury–cadmium telluride detector. A total of 400 scans at a resolution of 8 cm^{-1} were recorded at room temperature. (A) In KCl–HCl buffer, pH 1.8, at a concentration of 0.275 mg/mL. (B) In KCl–HCl buffer, pH 2.25, at a concentration of 1.275 mg/mL.

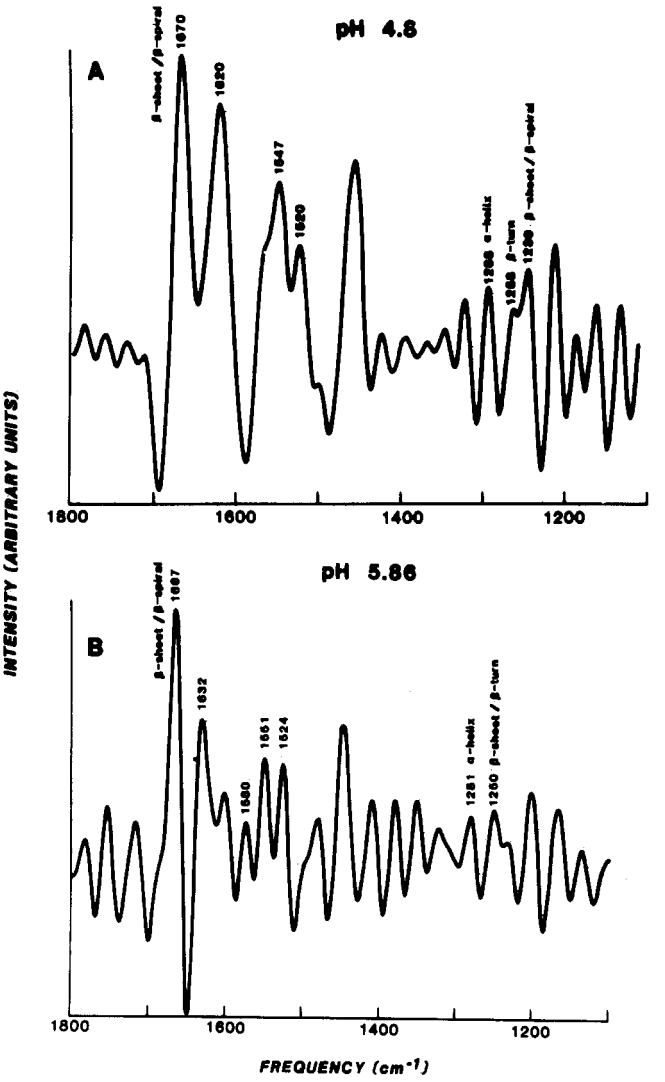

FIGURE 3: Resolution-enhanced FT-IR spectra of bovine amelogenin in solution at pH 4.8 and pH 5.68. Experimental conditions are the same as those in Figure 2. (A) In acetate buffer, pH 4.8, at a concentration of 0.275 mg/mL. (B) In piperazine buffer, pH 5.68, at a concentration of 0.175 mg/mL.

Preliminary Studies on the Effect of Ca^{2+} Binding on the Secondary Structure of Amelogenin in Solution. In view of the important role that the phosphorylated protein amelogenin is thought to assume in the mineralization of enamel (Glimcher, 1979; Strawich & Glimcher, 1980, 1985), we have carried out preliminary studies, using CD spectroscopy, on the effect of Ca^{2+} on its secondary structure. In addition to the neutral carbonyl groups which may play a role in chelating Ca^{2+} (Urry, 1971; Renugopalakrishnan & Urry, 1978; Renugopalakrishnan & Walter, 1980; Renugopalakrishnan et al., 1981), the abundance of Glu residues and the *o*-phosphoserine residue should also be expected to confer potential Ca^{2+} binding sites to amelogenin. The CD spectra of amelogenin in solution at pH 7.2, in the presence of increasing amounts of Ca^{2+} introduced as $CaCl_2$, are presented in Figure 1A. The CD spectral data and the percentage secondary structure derived from them by using the procedure of Chang et al. (1978) are listed in Table I. At 4 μM Ca^{2+} concentration, the CD spectrum appears qualitatively similar to the free amelogenin CD spectrum with little change in the calculated percent of secondary structures. However, at 8 μM Ca^{2+} concentration, the CD spectra appear somewhat different with a slight reduction in percent α-helix, percent β-turn, and percent "unordered" structures by 2.5%, 3.5%, and 1.5%, respectively, which are within experimental error. On the other hand, the percent β-sheet is found to increase by 8.5%. At 16 μM Ca^{2+}, the CD spectrum is observed to be quite different with significant reduction in percent α-helix, percent β-turn, and percent unordered structures by 9.5%, 12.5%, and 9%, respectively. The percent β-sheet increases significantly by 31%. The minimum in wavelength is also shifted to 211 nm. The effect of Ca^{2+} on the amelogenin secondary structure at pH 1.6 is found to be negligible and within the margin for experimental error (Figure 1B).

Discussion

Solution Phase. The CD spectral data of bovine amelogenin solutions at pH 7.2 and 1.6 are shown in panels A and B, respectively, of Figure 1, and the CD spectral data are tabulated in Table I. At pH 7.2, amelogenin exhibits a negative trough at 208 nm with a molar ellipticity, $[\theta]$, of −11 666 deg·cm^2·$dmol^{-1}$ and a positive peak at ~196 nm with a molar ellipticity, $[\theta]$, of 1167 deg·cm^2·$dmol^{-1}$. The CD spectrum at pH 7.2 resembles a class C β-turn CD spectrum in the theoretical analysis of CD spectra by Woody (1974) [see also Crisma et al. (1984) and Hollosi et al. (1985)]. At pH 1.6, however, the CD spectra (Figure 1B) are quite different and

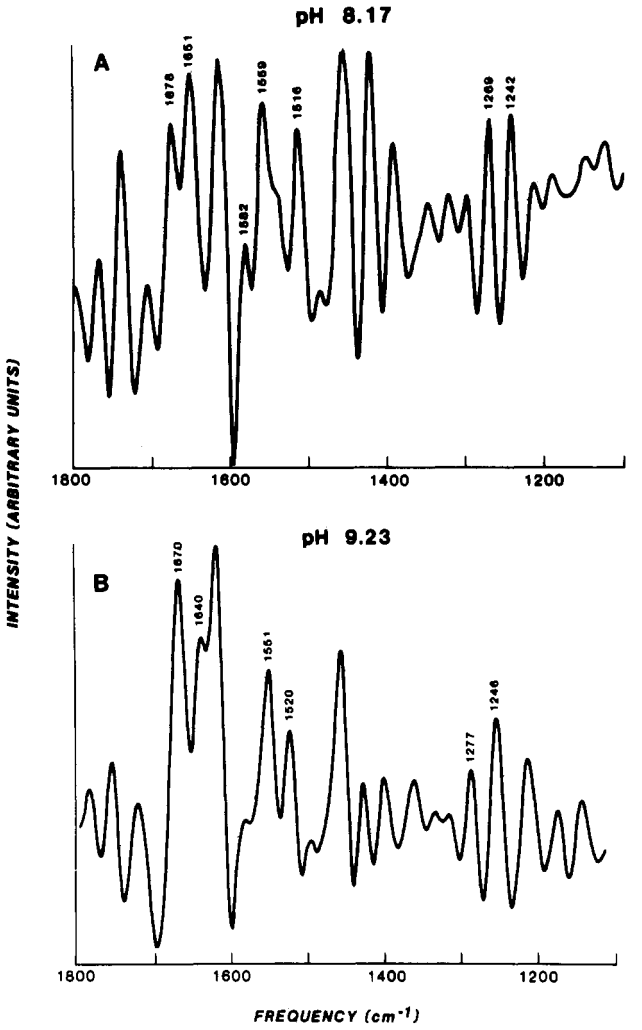

FIGURE 4: Resolution-enhanced FT-IR spectra of bovine amelogenin in solution at pH 8.17 and 9.23. Experimental conditions are the same as those in Figure 2. (A) In NH_4HCO_3 buffer, pH 8.17, at a concentration of 0.035 mg/mL. (B) In Tris buffer, pH 9.23, at a concentration of 0.069 mg/mL.

resemble superficially an unordered structure without readily discernible elements of secondary structure (Yang et al., 1976). The CD spectrum at pH 1.6 displays a negative trough at 203 nm with a molar ellipticity, $[\theta]$, of $-11\,000$ deg·cm^2·dmol^{-1}, and the positive peak is abolished. All CD spectral data obtained in this study were analyzed to derive the fraction of secondary structures using an algorithm described by Chang et al. (1978). The above algorithm was developed on the basis of observed CD spectra of proteins and calculates the fraction of α-helix, β-sheet, β-turns, and unordered structures from the molar ellipticity, $[\theta]$, using values of reference conformations derived from CD spectra of 15 proteins with well-established secondary structures (Chang et al., 1978). From the CD spectrum of amelogenin at near-neutral pH (Figure 1A), the following fractions of secondary structures were calculated: 16% α-helix, 30% β-sheet, 21% β-turn, and 33% unordered structure. At pH 1.6, the calculations showed 0% α-helix, 58% β-sheet, 3.5% β-turn, and 38.5% unordered structures. The estimation of β-turn fractions by the algorithm developed by Chang et al. (1978) is not entirely satisfactory due to the discrepancies between the theoretical CD calculations of Woody (1974) and experimentally observed CD spectra of β-turn structures (Hollosi et al., 1985), and, hence, the fractions derived here for the amelogenin are to be regarded as tentative and of qualitative significance only.

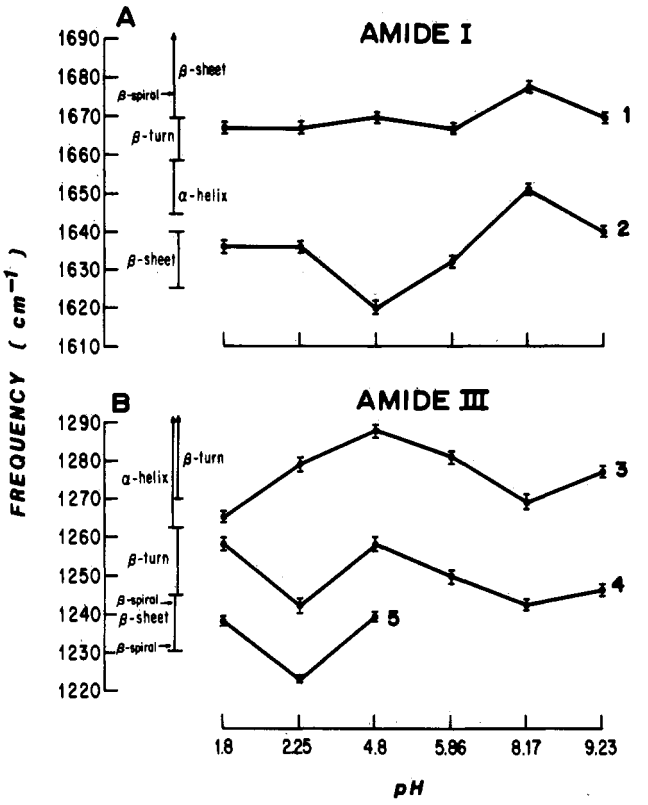

FIGURE 5: Resolution-enhanced FT-IR pH titration showing the variation of amide I and II frequencies in cm^{-1} as a function of pH. The usual range of α-helix, β-sheet, β-turn, and β-spiral structures is indicated on the abscissa.

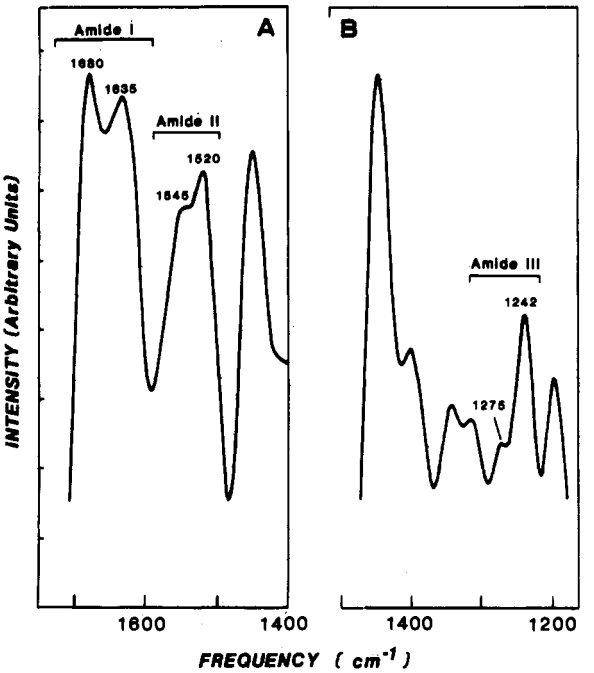

FIGURE 6: Fourier transform infrared deconvoluted spectra of bovine amelogenin in the solid state at room temperature. The spectra were recorded on a Digilab FTS-14 Fourier transform infrared spectrometer at a resolution of 4 cm^{-1}. A total of 400 scans were accumulated for each spectra. The sample was contained in a diamond cell. (A) Amide I and II region, 1400–1700 cm^{-1}. (B) Amide III region, 1100–1500 cm^{-1}.

The positive peak exhibited by bovine amelogenin at 196 nm at pH 7.2 (Figure 1A) is qualitatively somewhat similar to the 205-nm positive peak observed in the CD spectrum of the polytetrapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Gly)_n$ (Urry et al., 1974),

which has been demonstrated by ^1H NMR studies (Khaled et al., 1976) to contain a β-turn between the Val_1 C=O and the Gly_4 NH groups and forms a β-spiral in solution (Urry & Long, 1976). Due to the presence of β-sheet structures in bovine amelogenin, the negative troughs of the CD spectra of bovine amelogenin and the polytetrapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Gly)_n$ probably do not correspond to each other. The CD spectrum of amelogenin at pH 7.2 bears greater similarity to the human 7S myeloma IgG (Jirgenson, 1973) which is known to be a nonhelical protein containing β-sheets and β-turns. The CD spectrum of amelogenin at pH 7.2 also bears similarities to CD spectra of α-chymotrypsin, elastase, and concanavalin A (Yang et al., 1976) which share a common secondary structural feature—low α-helical content and high β-sheet and β-turn contents.

The 1667 and 1632 cm^{-1} bands at pH 5.86 (Figure 3B) are tentatively assigned to a composite of β-sheet (Renugopalakrishnan et al., 1985b) and repetitive β-turn structures since they are also observed in aqueous solutions of the polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ (G. J. Thomas, Jr., and D. W. Urry, unpublished results) and the neuromuscular blocking agent anthopleurin A (Ishizaki et al., 1979) and are within the range theoretically predicted for β-turn structures (Bandekar & Krimm, 1979). Interestingly, gramicidin A manifests a strong IR-active amide I band at 1638 cm^{-1}, with a shoulder around $\sim$1680 cm^{-1} (Naik & Krimm, 1984), which is quite close to the amide I frequency of 1633 $\pm$ 1 cm^{-1} reported by Urry et al. (1983). Urry et al. (1983) have discussed the relevance of the observed amide I frequency to the single-stranded β^6-helix, present in gramicidin A, which can interconvert to an anti-β^6-spiral structure, representing the conducting and nonconducting species in ion-conducting channels. On lowering the pH to 4.8 (Figure 3A), the higher frequency component of the amide I band shifts to 1670 cm^{-1} whereas the lower frequency component shifts to 1620 cm^{-1}. The 1670 cm^{-1} band could arise from a composite of β-sheet and repetitive β-turn structures. The secondary structural interpretation of the 1620 cm^{-1} band remains to be resolved, and it may well be due to water bending modes.

However, on further lowering the pH to 2.25 and 1.8 (Figure 2A,B), the amide I band retains its doublet nature with frequencies of 1667 and 1636 cm^{-1}, although the 1636 cm^{-1} band at pH 1.8 is a shoulder on the $\sim$1620 cm^{-1} band. The conformational assignments of the amide I bands at 1667 and 1636 cm^{-1} follow the same arguments advanced for the amide I bands at pH 4.8 and 5.86 above.

When the pH is raised to 8.17, the amide I band shifts to a higher frequency of 1678 cm^{-1} with a lower frequency component at 1651 cm^{-1} (Figure 4A). The higher frequency component at 1678 cm^{-1} is quite close to the amide I band at 1673 cm^{-1} observed in the synthetic polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ which is an indication of a stabilization of the repetitive β-turn structure. Unfortunately, there is no indication of β-sheet structure at pH 8.17 due to the absence of bands in the 1630–1640 cm^{-1} range, although it is possible that the β-turn vibrational modes and antiparallel β-sheet vibrational modes, A and B_1 (Nevskaya & Chirgadze, 1976a), have coalesced into a composite band. At a still higher pH of 9.23 (Figure 4B), the amide I band shifts to a lower frequency of 1670 cm^{-1} with a shoulder at 1640 cm^{-1}. The secondary structural implications of these results are not completely clear at this time.

Amide III bands for repetitive structure in $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ occur at 1242 and 1232 cm^{-1} (G. J. Thomas, Jr., and D. W. Urry, unpublished results). The former is not too different from the amide III band observed in anthopleurin A (Ishizaki et al., 1979) and is within the range observed theoretically for β-turn structures (Bandekar & Krimm, 1979). The amide III bands for amelogenin at pH 4.8 and 5.86 (Figure 3A,B) occur as doublets with shoulders. The band at 1239 cm^{-1} at pH 4.8 does lend further support to the conclusions reached on the basis of the amide I band at the same pH. The amide III band at 1288 cm^{-1} at pH 4.8 suggests the presence of an α-helical structure in bovine amelogenin. From Chou–Fasman calculations on the secondary structure of amelogenin, short segments, i.e., $Ser_{55}\text{-}Gln_{56}\text{-}Gln_{57}$ and $His_{62}\text{-}Ala_{63}\text{-}Gly_{64}\text{-}Gln_{65}$, do exhibit $\langle P_\alpha \rangle$ values of 0.997 and 1.26, respectively, but they are too short to nucleate α-helix formation. The segment $Gly_{44}\text{-}Trp_{45}\text{-}Leu_{46}\text{-}His_{47}\text{-}His_{48}\text{-}Gln_{49}\text{-}Ile_{50}$, with a $\langle P_\alpha \rangle$ value of 1.12 probably under the influence of pH, can assume an α-helical structure. Nevertheless, the contribution of the α-helical structure to the total secondary structures of bovine amelogenin is still minimal. The amide III band at pH 5.86 (Figure 3B) occurs at 1250 cm^{-1} with a lower frequency shoulder around 1240 cm^{-1} and at 1281 cm^{-1}. The above bands can again be attributed to a composite of β-sheet and repetitive β-turn structures.

At higher pH values of 8.17 and 9.23, the composite β-sheet and repetitive β-turn structures continue to be the dominant secondary structure as evidenced by the presence of 1242 and 1246 cm^{-1} bands.

The recently reported primary structure of bovine amelogenin by Takagi et al. (1984) and the physical–chemical studies reported on bovine amelogenin in solution and in the solid state discussed here shed some insight on its secondary structure.

Chou–Fasman calculations on dephosphorylated bovine amelogein indicate that residues Ile_{13} through Leu_{20}($\langle P_\beta \rangle$ = 1.27), Trp_{25} through Arg_{31} ($\langle P_\beta \rangle$ = 1.03), Tyr_{37} through Tyr_{39} ($\langle P_\beta \rangle$ = 1.24), and Gly_{44} through Ile_{50} ($\langle P_\beta \rangle$ = 1.12) show a distinct preference for the β-sheet structure. In the N-terminal region, His_6 through Tyr_{12} forms two strong contiguous β-turns with probabilities of 4.3×10^{-4} and 10×10^{-4}, respectively. Similarly, Thr_{21} through Lys_{24}, Gln_{40} through Gly_{43}, Ile_{51} through Val_{54}, Thr_{58} through Asp_{61}, Ile_{70} through Val_{73}, Gln_{77} through Gln_{83}, Val_{88} through Glu_{91}, and Gln_{93} through Leu_{96} are likely to form β-turns with widely varying probabilities of occurrence. The residues His_{62} through Gln_{65} may form a very weak α-helical structure, as shown in Figure 1, since a minimum of four residues has been assumed to nucleate the α-helix (Chou & Fasman, 1978). Pauling et al. (1951) originally proposed an α-helical structure with 3.7 residues per turn, but the segment His_{62} through Gln_{65} is too short to form an α-helical structure and hence may remain unordered. The Pro-Pro sequences, $Pro_4\text{-}Pro_5$, $Pro_{66}\text{-}Pro_{67}$, $Pro_{140}\text{-}Pro_{141}$, $Pro_{151}\text{-}Pro_{152}$, $Pro_{154}\text{-}Pro_{155}$, and $Pro_{159}\text{-}P_{160}$, are expected to assume a conformationally restricted polyproline-type structure (Venkatachalam et al., 1975). At the level of Chou–Fasman calculations, employed in this study, we have tacitly assumed trans peptide bonds around Pro–Pro junctions. At the present level of understanding of amelogenin structure, it makes no difference whether the peptide bonds are trans-trans or cis-cis, or the other combinations thereof. Therefore, out of the several choices available, the trans-trans conformation was arbitrarily assumed. The residues Gln_{112} through His_{139}, with the generic structure $(Gln\text{-}Pro\text{-}X)_9\text{-}His$, form a series of nine repetitive β-turns with Pro and X as the $i + 1$ and $i + 2$ residues and Gln as the hinge residue, nucleating the β-turn. The Chou–Fasman algorithm does not permit predictions of a β-spiral structure. However, both the

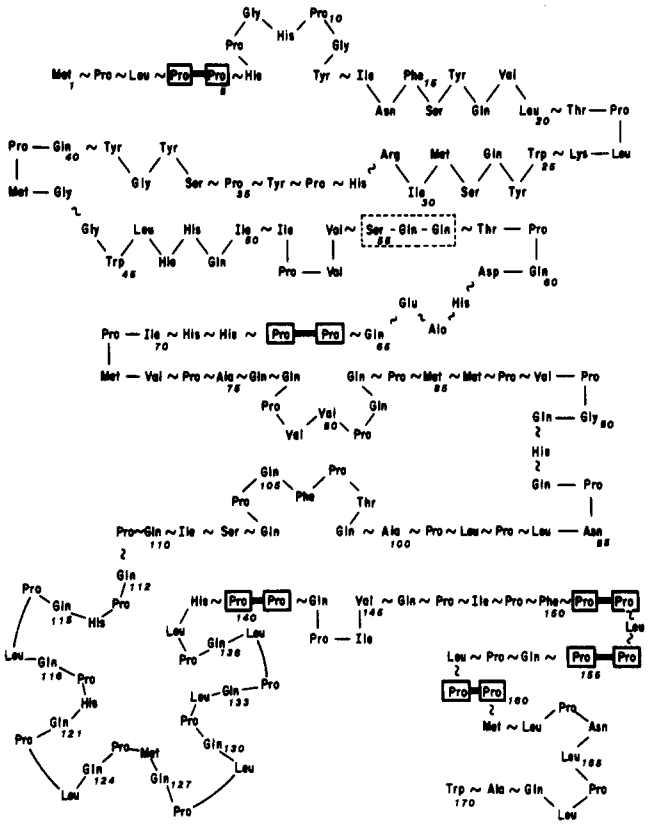

FIGURE 7: Predicted secondary structure of dephosphorylated bovine amelogenin from the primary structure reported by Takagi et al. (1984), using the Chou–Fasman algorithm. (∧∧∧), (⊓), and (∼·∼) represent β-sheet, β-turn, and "unordered" structures. Ser(P) occurs at position 16. Polyproline-type structures occurring in Pro-Pro segments are shown boxed in boldface. A trans-trans conformation for peptide bonds was tacitly assumed in the predicted secondary structure.

experimental data and the Chou–Fasman algorithm strongly suggest that there are regions already outlined which contain repetitive β-turns. While it is not possible to conclude from these data the unique conformation which occurs from Gln_{112} to His_{139}, there are a number of possible configurations, but the similarity between the spectra obtained from amelogenin and those obtained from several peptides (Urry, 1982, 1983) leads us to suggest the β-spiral as a reasonable choice. The predicted secondary structure of bovine amelogenin is presented in Figure 7.

The calculated secondary structure of amelogenin is shown in Figure 7. Since the secondary structure contains an unusually large number of β-turns, the probabilities (p_t) of the occurrence of 25 β-turns are shown in a histogram (Figure 8). The average probability of β-turn occurrence, $\langle p_t \rangle = 0.55 \times 10^{-4}$, is marked in Figure 8.

The total probability of bend occurrence for a cluster of four residues is calculated from the expression [by Chou & Fasman (1978)]:

$$p_t = f_i f_{i+1} f_{i+2} f_{i+3}$$

where f_i's are bend frequencies whose values were adapted from the same authors. The total probability, p_t, and not individual residue probabilities, for a cluster of four residues is plotted against the residue number.

The presence of β-sheet structures in the enamel proteins of developing teeth in the solid state has been demonstrated by X-ray diffraction (Glimcher et al., 1961) and electron diffraction (Traub et al., 1985), and some of the implications of this structure for its possible physical–chemical and biological functions have been discussed (Glimcher, 1979, 1981, 1984).

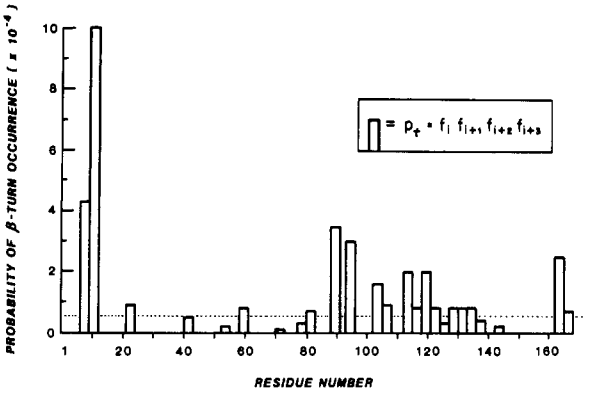

FIGURE 8: Probability of occurrence of 25 β-turns plotted against residue numbers. The probability of occurrence (p_t) was calculated as the product of bend frequencies ($f_i f_{i+1} f_{i+2} f_{i+3}$) determined from frequencies of bends of Chou and Fasman (1978). The average probability of β-turn occurrence, $\langle p_t \rangle = 0.55 \times 10^{-4}$, is indicated in the figure. The total probability, p_t, for a cluster of four residues is plotted against the residue numbers.

The secondary structure of amelogenin in solution seems to consist principally of β-sheet–repetitive β-turn structure both at physiological pH and at a wide range of pH values. However, the two methods, as previously noted in the case of phosvitin (Renugopalakrishnan et al., 1985b), are not in absolute accord with each other. CD studies predict that, at neutral pH, there will be a larger percentage of β-sheet structure than β-turn structure, whereas at pH 1.6, the proportion of β-turn structure is negligible. These predictions are not in agreement with Chou–Fasman and FT-IR spectroscopic studies in solution. As previously noted, the CD spectral pattern at pH 7.2 of bovine amelogenin does bear resemblance to the CD spectra of the polytetrapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Gly)_n$ in methanol which argues for the presence of repetitive β-turns in bovine amelogenin. Further work is required to resolve the discrepancies between the two methods.

Solid State. The two broad bands in the FT-IR deconvoluted spectrum of bovine amelogenin (Figure 6A), at 1680 and 1635 cm^{-1}, respectively, are indicative of a β-sheet structure based on amide I bands of polyserine (1668 cm^{-1}) (Koenig & Sutton, 1971), carbonic anhydrase, (40% β-sheets), bovine pancreatic trypsin inhibitor (37% β-sheets, 1667 cm^{-1}) (Tu, 1982; Seaton, 1983), phosvitin (Renugopalakrishnan et al., 1985b), and polypeptide neurotoxins from snake venom (Takamatsu et al., 1980).

However, as discussed in the previous section, the presence of a large number of Pro residues, i.e., ∼300 residues per 1000, results in the occurrence of several β-turns including contiguous β-turns in the C-terminal region. Recently, the Raman spectra of a polypentapeptide model $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ of tropoelastin (G. J. Thomas, Jr., and D. W. Urry, unpublished results), which occurs in approximately 10% of swine tropoelastin and anthopleurin A (Ishizaki et al., 1979), were investigated and found to manifest similar amide I frequencies as bovine amelogenin.

The monomer of the polypentapeptide $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5)_n$ has been shown from ^1H NMR and conformational energy calculations to form a type II β-turn structure involving the Val_1 C=O and Val_4 N–H groups (Renugopalakrishnan et al., 1978). The presence of a β-turn has also been corroborated by ^{13}C NMR studies (Urry et al., 1975; Urry & Long, 1976). The polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ is stabilized by repetitive β-turns which coil into a

β-spiral structure, a unique protein structural type described by Urry (1972).

Conformational energy calculations, NMR (Venkatachalam et al., 1981) studies, and X-ray crystallographic data (Cook et al., 1980) from the cyclic correlate of the pentapeptide Val-Pro-Gly-Val-Gly (the cyclododecapeptide) have demonstrated the β-turn structure. On the basis of the Chou–Fasman calculations of the amino acid sequence of Takagi et al. (1984) and the similarities of amide I frequencies from recent Raman studies of the polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$, cyclo-$(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_3$, and anthopleurin A (Ishizaki et al., 1979) as well as similarities with the theoretical β-turn frequencies (Bandekar & Krimm, 1979), we are led to tentatively conclude that the 1680 cm^{-1} band originates from a composite of β-sheet and repetitive β-turn structures. The above conclusion is further corroborated from the similarity of the intense amide III mode at 1242 cm^{-1} (Figure 6B) with the 1239 cm^{-1} band observed in the polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ which has been shown to form a β-spiral structure (Urry & Long, 1976; Urry, 1982; Urry et al., 1983).

The experimental studies of bovine amelogenin suggest an unusual secondary structure in the solid state and in solution which bears some similarities to other β-sheet-containing proteins. However, the presence of a large number of Pro residues ($\sim$300 Pro residues per 1000 residues) argues against a total β-sheet structure and indicates the presence of β-turns interspersed amidst β-sheet structure. The presence of β-turns is in accord with Chou–Fasman calculations (Chou & Fasman, 1978) based on the recently reported sequence of bovine amelogenin (Takagi et al., 1984). The presence of contiguous β-turns in amelogenin is reminiscent of secondary structures of polypeptides of tropoelastin proposed by Urry et al. (1982) and references cited therein. However, the secondary structural model of amelogenin, especially the β-spiral conformation proposed here, is to be regarded as tentative at this stage.

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Intramolecular Distance Measurements in α-Lactalbumin†

Giovanni Musci and Lawrence J. Berliner*
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210
Received January 6, 1986; Revised Manuscript Received March 18, 1986

ABSTRACT: The distance between the calcium site (*site I*) and the zinc site (*site II*) in α-lactalbumin was estimated from Forster energy-transfer measurements between donor Eu(III) [or Tb(III)] at *site I* and acceptor Co(II) at *site II* to be 11.5 ± 1.5 Å. Intersite distances were also measured between the bis-ANS [4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]] binding locus and cobalt at *site II* (13.6 ± 1.0 Å), between bis-ANS and a fluorescein moiety covalently bound to Met-90 (33.5 ± 3.0 Å), and between Met-90 (fluorescein) and cobalt at *site II* (16.7 ± 1.0 Å). The apparent K_d for cobalt binding to *site II* agreed well with the value measured previously by intrinsic fluorescence [Murakami, K., & Berliner, L. J. (1983) *Biochemistry 22*, 3370–3374]. A Zn(II) titration of Eu(III)–α-lactalbumin reconfirmed that both *sites I* and *II* can be occupied simultaneously [Musci, G., & Berliner, L. J. (1985) *Biochemistry 24*, 3852–3856], since the lanthanide fluorescence was unaffected.

α-Lactalbumin (α-LA)[1] is a low molecular weight protein found in mammalian milk that plays a crucial role in the biosynthesis of lactose. Furthermore, it has been shown that α-LA is a calcium-binding protein with K_d = 0.2–3 nM (Permyakov et al., 1981, 1985; Murakami et al., 1982). A second cation site, *site II*, is specific for Zn(II), Co(II), Cu(II), and Al(III) (Murakami & Berliner, 1983; Musci & Berliner, 1985a). Specific cation binding to α-LA is important in modulating protein conformation and hence its activity in the lactose synthase complex (Murakami & Berliner, 1983; Musci & Berliner, 1985a,b). A hydrophobic site was found from fluorescence studies with the apolar dye bis-ANS by Musci and Berliner (1985a). The affinity of α-LA for the fluorophore was dependent on the state of metal-ion binding. The precise location of this hydrophobic binding region is important to our understanding of the α-LA–galactosyltransferase interaction, as suggested from kinetic result studies (Berliner et al., 1984) and by the inhibition of lactose synthesis by bis-ANS (Musci

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* Author to whom correspondence should be addressed.

[1] Abbreviations: α-LA, α-lactalbumin; bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]; EDTA, ethylenediaminetetraacetic acid; EF, enhancement factor for bis-ANS binding to α-LA (i.e., the intensity ratio of the binary complex to that of the free dye); ESR, electron spin resonance; GT, galactosyltransferase; IAF, (iodoacetamido)fluorescein; NMR, nuclear magnetic resonance; UDP-Gal, uridine 5′-diphosphate galactose; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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