Formation of δ_1-Acetoxytryptophan-62 in the Oxidation of Tryptophan-62 of Hen Egg-White Lysozyme by *N*-Bromosuccinimide in Acetate Buffer†

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ABSTRACT: The reaction of equimolar amounts of *N*-bromosuccinimide and hen egg-white lysozyme in acetate buffer, under the conditions of Hayashi et al. (Hayashi, K., Imoto, T., Funatsu, G., and Funatsu, M. (1965), *J. Biochem. (Tokyo) 58*, 227), yields a protein mixture that has a time-dependent ^{13}C-NMR spectrum. The initial natural-abundance ^{13}C-NMR spectrum indicates the presence of about equal amounts of [oxindolealanine-62]lysozyme and [δ_1-acetoxytryptophan-62]lysozyme. The latter converts to [oxindolealanine-62]lysozyme with a half-life of about 2 days at 25 °C and pH 3.9. Two observations indicate that the source of the acetyl group of δ_1-acetoxytryptophan-62 is the acetate buffer. First, the spectrum of a lysozyme sample treated with *N*-bromosuccinimide in the presence of [1-^{13}C]acetate yields a very strong acetyl ester carbonyl resonance. The time dependence of the intensity of this resonance yields a half-life of 44 h for [δ_1-acetoxytryptophan-62]lysozyme. Second, the initial natural-abundance ^{13}C-NMR spectrum of a lysozyme sample treated with *N*-bromosuccinimide in the absence of acetate indicates essentially complete conversion of tryptophan-62 into oxindolealanine.

N-Bromosuccinimide has been used extensively as a reagent in studies of tryptophan residues of proteins (Spande and Witkop, 1967a–d; Spande et al., 1970; Kronman and Robbins, 1970). In particular, *N*-bromosuccinimide has been used to selectively oxidize tryptophan residues of native proteins, in attempts to determine whether or not these residues play a role in the structure and/or function of the proteins (Spande and Witkop, 1967b,c; Spande et al., 1970). Probably the most widely quoted example of a specific chemical modification of a protein by *N*-bromosuccinimide is the oxidation of Trp-62 of hen egg-white lysozyme. Hayashi et al. (1965) showed that addition of an equimolar amount of *N*-bromosuccinimide to lysozyme in 0.1 M acetate buffer at pH 4.5 and 10 °C caused the selective oxidation of Trp-62. Their spectrophotometric data were consistent with the formation of an oxindolealanine[1] residue (see Hartdegen, 1967). On the basis of optical rotation data, Takahashi et al. (1965) concluded that [oxindolealanine-62]lysozyme has essentially the same conformation as intact lysozyme. This property of the modified protein, together with its lack of enzymatic activity and simplicity of preparation, have encouraged extensive studies of this species (Imoto et al., 1972).

Recently, we compared the natural-abundance ^{13}C-NMR spectrum of [oxindolealanine-62]lysozyme with that of intact lysozyme, in an attempt to identify the ^{13}C resonances of Trp-62 in the spectrum of the latter (Oldfield et al., 1975b). In the course of our studies, we noticed that a few resonances in the ^{13}C-NMR spectra of freshly prepared samples of the modified protein exhibited a time-dependent behavior. In this paper, we present a detailed study of this time dependence, and we show that a transient species persists for several days after completion of the treatment with *N*-bromosuccinimide. We also show that the transient species is [δ_1-acetoxytryptophan-62]lysozyme.

Experimental Procedure

Materials. Hen egg-white lysozyme from Miles Laboratories, Inc., Kankakee, Ill. (six times crystallized) was dialyzed against deionized water at pH 3. Lysozyme from Sigma Chemical Co., St. Louis, Mo. (grade I) was purified by chromatography on DEAE-Sephadex.[2] *N*-Bromosuccinimide from Sigma was recrystallized three times from water. Sodium [1-^{13}C]acetate (nominally 90% ^{13}C enriched) was purchased from Merck and Co., Inc., Rahway, N.J., and used as received. The purity of this material was checked by elemental analysis and ^{13}C NMR. The isotopic enrichment measured by mass spectrometry was 90.6%. 2-Bromo-4-methylphenol and 2,6-dibromo-4-methylphenol were obtained from Aldrich Chemical Co., Milwaukee, Wis. Guanidine hydrochloride (ultrapure grade) was obtained from Schwarz-Mann, Orangeburg, N.Y.

Methods. In most experiments hen egg-white lysozyme was treated with an equimolar amount of *N*-bromosuccinimide according to the procedure of Hayashi et al. (1965), except that after completion of the reaction the protein was dialyzed against frequent changes of deionized water (at pH 3.2–3.4 and 4 °C) for 12–21 h, and concentrated by ultrafiltration at 4 °C in a Model 52 or 402 ultrafiltration cell (Amicon Corp., Lexington, Mass.) equipped with a UM-10 or PM-10 membrane. In the experiment in which acetate buffer was omitted, the pH of the reaction mixture was maintained at 4.5 by adding dilute NaOH for 30 min after addition of *N*-bromosuccinimide. The protein was then dialyzed and concentrated as above. One sample of [oxindolealanine-62]lysozyme was prepared according to Hayashi et al. (1965) and dialyzed as specified by those authors, viz., against running water (20–21°) for 2 days, then against deionized water (4 °C) for 2 days. In this

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[1] We use the term oxindolealanine to indicate β-(3-oxindolyl)-L-alanine.

[2] Abbreviations used are: Me_4Si, tetramethylsilane; NOE, nuclear Overhauser enhancement; DEAE, diethylaminoethyl.

case, the modified protein was concentrated by ultrafiltration at room temperature. All pH measurements were carried out at room temperature. Protein concentrations were determined spectrophotometrically with the use of the following values for $E_{281}^{1\%}$: lysozyme, 25.5 (Imoto et al., 1972); [oxindolealanine-62]lysozyme, 22.0 (Hayashi et al., 1965); [δ_1-acetoxytryptophan-62]lysozyme, 25.5 (assumed to be the same as for lysozyme).

Natural-abundance ^{13}C Fourier transform NMR spectra were obtained at 15.18 MHz with the use of 20-mm spinning sample tubes (Allerhand et al., 1973a), as described previously (Allerhand et al., 1973b; Oldfield et al., 1975a). A spectral width of 3787.9 Hz was used. Time-domain data were accumulated in 8192 addresses of a Nicolet 1085 computer. Fourier transformation was done on 16 384 time-domain addresses by placing 8192 addresses with a zero value at the end of each block of accumulated data points. Most spectra were recorded under conditions of noise-modulated off-resonance proton decoupling (Wenkert et al., 1969), as described previously (Oldfield et al., 1975a,b). Broad methine aromatic carbon resonances were removed from the spectra of proteins by means of the convolution-difference procedure (Campbell et al., 1973), as described previously (Oldfield et al., 1975b). The values of τ_1, τ_2, and K (defined as in Campbell et al., 1973) were 0.36 s, 0.033 s, and 0.9, respectively. Chemical shifts were obtained digitally, and are reported in parts per million downfield from the ^{13}C resonance of Me_4Si. Estimated accuracy is ±0.05 ppm for fully resolved peaks and decreases to ±0.1 ppm with decreasing resolution. Dilute aqueous dioxane (at 67.8_6 ppm downfield from external Me_4Si) was used as an internal reference for all aqueous samples, except in Figure 1B, where external ethylene glycol (at 64.3 ppm) was used. The chemical shifts of small molecules dissolved in dioxane were referenced to the ^{13}C resonance of the solvent (at 67.5_8 ppm downfield from external Me_4Si).

The T_1 value of the resonance of the ^{13}C-enriched carbonyl of the sample of [δ_1-acetoxytryptophan-62]lysozyme prepared with the use of 90% enriched [1-^{13}C]acetate was measured by progressive saturation (Freeman and Hill, 1971). Estimated accuracy is ±20%. The nuclear Overhauser enhancement (NOE) of this resonance was taken as the ratio of intensities in spectra recorded with full proton decoupling (Oldfield et al., 1975a) and with gated proton decoupling (Freeman et al., 1972). The condition of no proton decoupling was achieved by shifting the proton irradiation 70 kHz off resonance and switching off the noise modulation. Estimated accuracy of the NOE is ±20%. The T_1 and NOE values were obtained at 25 °C immediately after completion of the dialysis and ultrafiltration at 4 °C. Sixty-four accumulations per spectrum were used. The recycle times were 1.1, 2.2, 5.0, and 20 s for the T_1 measurement, and 20 s for the NOE determination. Integrated intensities were measured digitally.

Results and Discussion

Assignments in the Spectrum of [Oxindolealanine-62]-Lysozyme. In this paper we shall be concerned mainly with the relatively narrow resonances of *nonprotonated* aromatic carbons (Oldfield et al., 1975a). Most of the 28 nonprotonated aromatic carbons of native hen egg-white lysozyme yield resolved single-carbon resonances (Allerhand et al., 1973b). The observation of these resonances is facilitated by the use of noise-modulated off-resonance proton decoupling and application of the convolution difference procedure (Campbell et al., 1973) for the removal of the broad bands of methine aromatic carbon resonances (Oldfield et al., 1975b). Figure 1A shows the aromatic region (and the downfield edge of the carbonyl region) in the convolution difference proton-decoupled ^{13}C-NMR spectrum of intact lysozyme (Oldfield et al., 1975b). Figure 1B shows the corresponding spectral regions of a sample of [oxindolealanine-62]lysozyme prepared according to Hayashi et al. (1965). The product of the reaction was first dialyzed for 4 days (2 days at 20 °C and 2 days at 4 °C), and then stored for 5 days (at 4 °C) before the ^{13}C-NMR spectrum was recorded at 42 °C.

The 28 nonprotonated aromatic carbons of hen egg-white lysozyme give rise to peaks 4–26 of Figure 1A. The truncated band at about 158 ppm (peaks 1–3) arises from C^{ζ} of the 11 arginine residues (Oldfield et al., 1975b). The peak numbering system is that of Oldfield et al. (1975a,b). The chemical shifts of peaks 4–26 and some assignments to carbons of specific residues have been given elsewhere (Oldfield et al., 1975b). We have identified the resonances of C^{γ}, $C^{\delta 2}$, and $C^{\epsilon 2}$ of Trp-62 and -63 of hen egg-white lysozyme (Oldfield et al., 1975b; Norton, R. S., Childers, R. F., and Allerhand, A., unpublished results), but we have not assigned these resonances on a one-to-one basis to the two residues (Figure 1A). The peaks designated by numbers in the spectrum of the modified protein (Figure 1B) have the same chemical shifts (within our experimental error) as the corresponding peaks of intact lysozyme. The chemical shifts of the peaks designated by letters in Figure 1B are given in Table I.

Oxidation of a tryptophan residue to oxindolealanine converts C^{γ} from a nonprotonated aromatic carbon (Figure 2A) to a methine aliphatic carbon (Figure 2D). The effect on the aromatic region of the spectrum should be the disappearance of the C^{γ} resonance. Indeed, instead of peak 24 (at 110.8_4 ppm) and peak 25 (at 110.3_2 ppm) of the intact protein (Figure 1A), assigned to C^{γ} of Trp-62 and -63, but not on a one-to-one basis, the modified protein yields peak f, which has an intensity of 1.2 ± 0.2 carbons. The resonance at 181.8_9 ppm in the carbonyl region of the spectrum of the modified protein (peak a of Figure 1B) has an intensity of about one carbon, and no counterpart in the spectrum of the intact protein (Figure 1A). The chemical shift of peak a is very similar to reported values for C-2 (see Figure 2D) of the oxindole moiety in model compounds (Wenkert et al., 1970; Oldfield et al., 1975b).

In the region of resonances of $C^{\epsilon 2}$ of tryptophans and C^{γ} of phenylalanines (peaks 7–13 of Figure 1A), peak 8 and one of the two contributors to peak 12 of intact lysozyme (Figure 1A) have been assigned to $C^{\epsilon 2}$ of Trp-62 and -63, but not on a one-to-one basis. When going from intact lysozyme (Figure 1A) to the modified protein (Figure 1B), peak 12 loses the contribution of one of its two carbons, peak 8 disappears, peak 9 becomes more intense, and two new resonances appear (peaks b and c of Figure 1B). The sum of the intensities of peaks b and c corresponds to about one carbon. The chemical shifts of peaks b and c are similar to that of C-7a (see Figure 2D) of aqueous oxindole (Oldfield et al., 1975b), but they differ by nearly 4 ppm from the chemical shift of the nearest resonance of the intact protein (Figure 1A). We assign *both* peaks to $C^{\epsilon 2}$ of oxindolealanine-62 of the modified protein. The conversion of lysozyme into [oxindolealanine-62]lysozyme generates a new asymmetric center at C^{γ} of residue 62 (Figure 2D,E). We ascribe the splitting of the resonance of $C^{\epsilon 2}$ of oxindolealanine-62 to the presence of about equal amounts of the two diastereoisomers under the sample conditions of Figure 1B. Ohno et al. (1974) have reported that aqueous β-(3-oxindolyl)-L-alanine exists as a 1:1 mixture of the two diastereoisomers, and that the two isomers have different proton NMR spectra. However, we cannot rule out the possibility that the splitting

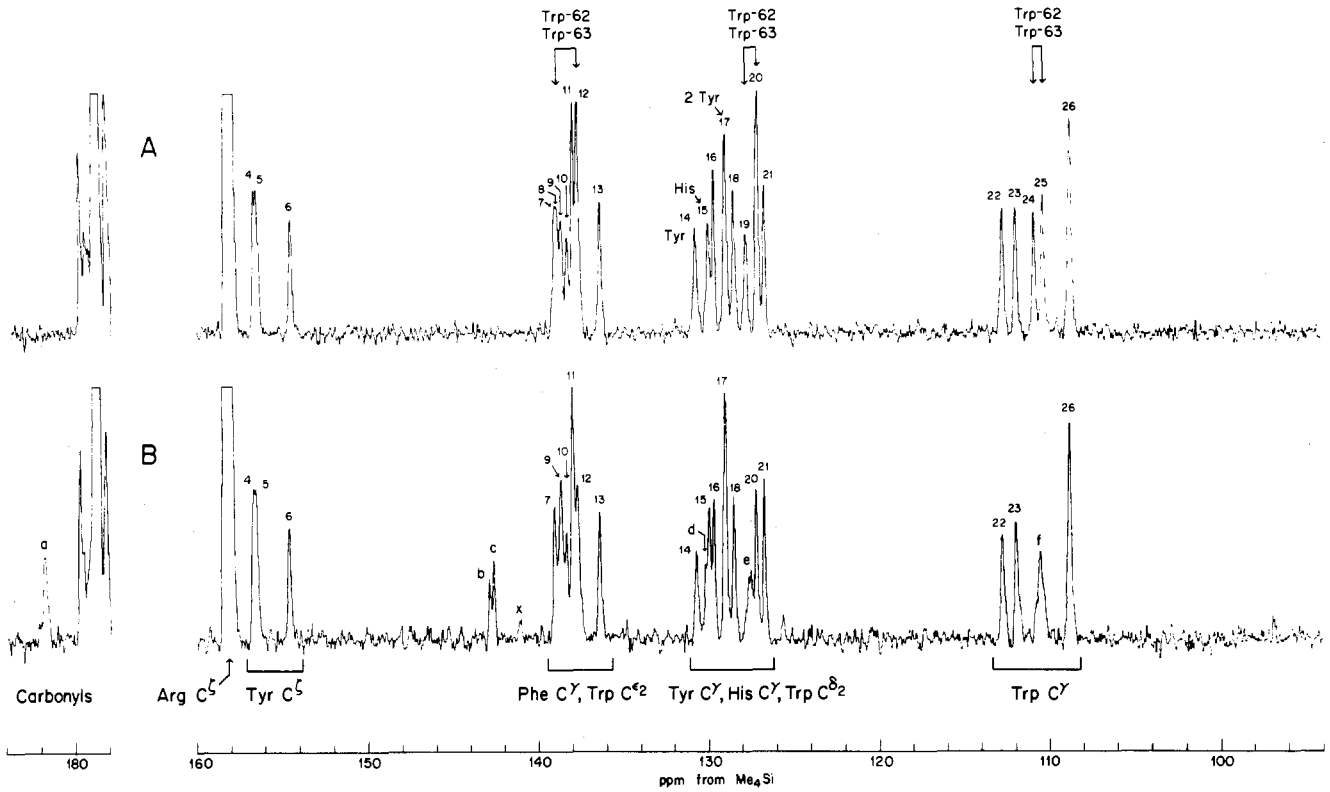

FIGURE 1: Region of aromatic carbons and downfield edge of the carbonyl region in convolution-difference natural-abundance ^{13}C-NMR spectra of hen egg-white lysozyme and [oxindolealanine-62]lysozyme. Each spectrum was recorded under conditions of noise-modulated off-resonance proton decoupling, with 65 536 accumulations and a recycle time of 2.2 s (40 h total time). Assignments are those of Oldfield et al. (1975b) and Table I. (A) 14.6 mM lysozyme in H_2O (0.1 M NaCl, pH 3.1, 44 °C). (B) 14.1 mM [oxindolealanine-62]lysozyme in H_2O (0.1 M NaCl, pH 3.1, 42 °C). After completion of the reaction with *N*-bromosuccinimide (in 0.1 M acetate buffer), the protein solution was dialyzed for 4 days (2 days at 20 and 2 days at 4 °C), stored for 5 days at 4 °C, concentrated by ultrafiltration for 3 h, and then kept at 42 °C for 22 h before the start of signal accumulation.

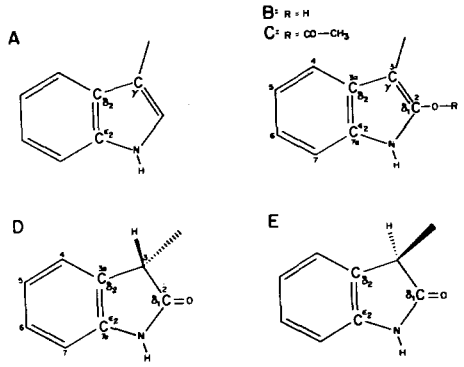

FIGURE 2: Structures. (A) Indolyl group of tryptophan. (B) 2-Hydroxyindolyl group of δ_1-hydroxytryptophan. (C) 2-Acetoxyindolyl group of δ_1-acetoxytryptophan. (D, E) Oxindolyl groups of the two diastereoisomers of oxindolealanine.

of the resonance of oxindolealanine-62 results from the existence of two protein conformations.

On the basis of the ^{13}C chemical shifts of tryptophan and oxindole (Oldfield et al., 1975b), we do not expect a major change in the chemical shift of $C^{\delta 2}$ when going from a tryptophan to an oxindolealanine residue. Peaks 19 and one-half of peak 20 in the spectrum of lysozyme (Figure 1A) have been assigned to $C^{\delta 2}$ of Trp-62 and -63, but not on a one-to-one basis. When going to the modified protein (Figure 1B), peaks 19 and one-half of peak 20 are replaced by peak e (intensity of about one carbon), peak d (intensity of about half a carbon), and a contribution of about half a carbon to peak 17. We assign peak e to Trp-63 and the two half-carbon resonances to residue 62 of the two forms of [oxindolealanine-62]lysozyme.

The chemical shifts of all aromatic residues other than Trp-62 and -63 of native lysozyme (Figure 1A) remain invariant when going to the modified protein (Figure 1B). This result, together with the fact that there is considerable chemical shift nonequivalence in Figure 1A as a consequence of protein folding (see below), strongly suggests that the overall conformation of lysozyme is not changed by the conversion of Trp-62 into an oxindolealanine residue. This conclusion is in agreement with the observations of Takahashi et al. (1965).

Denatured [Oxindolealanine-62]Lysozyme. Figure 3 shows the convolution difference ^{13}C-NMR spectra of intact lysozyme (Figure 3A) and [oxindolealanine-62]lysozyme (Figure 3B), both denatured with guanidinium chloride. In contrast to the situation with the native proteins (Figure 1), each residue now yields chemical shifts essentially independent of its position in the sequence. The resonances at 182.1_9 ppm and 142.3_6 ppm in the spectrum of the denatured modified protein (Figure 3B) are missing from the spectrum of the denatured intact protein (Figure 3A), and they have chemical shifts very similar to those of peaks a and b + c, respectively, in the spectrum of the native modified protein. We conclude that the chemical shifts of $C^{\delta 1}$ and $C^{\epsilon 2}$ of the oxindolyl group of the modified protein are not significantly affected by the secondary and tertiary structure. The δ_2-carbon of oxindolealanine-62 of the denatured modified protein yields either two sharp peaks coincident with the resonances of C^{γ} of Tyr and $C^{\delta 2}$ of Trp residues, or an undetected broad signal (Norton, R. S., and Allerhand, A., unpublished results).

Time-Dependent Behavior. The spectrum of Figure 1B was

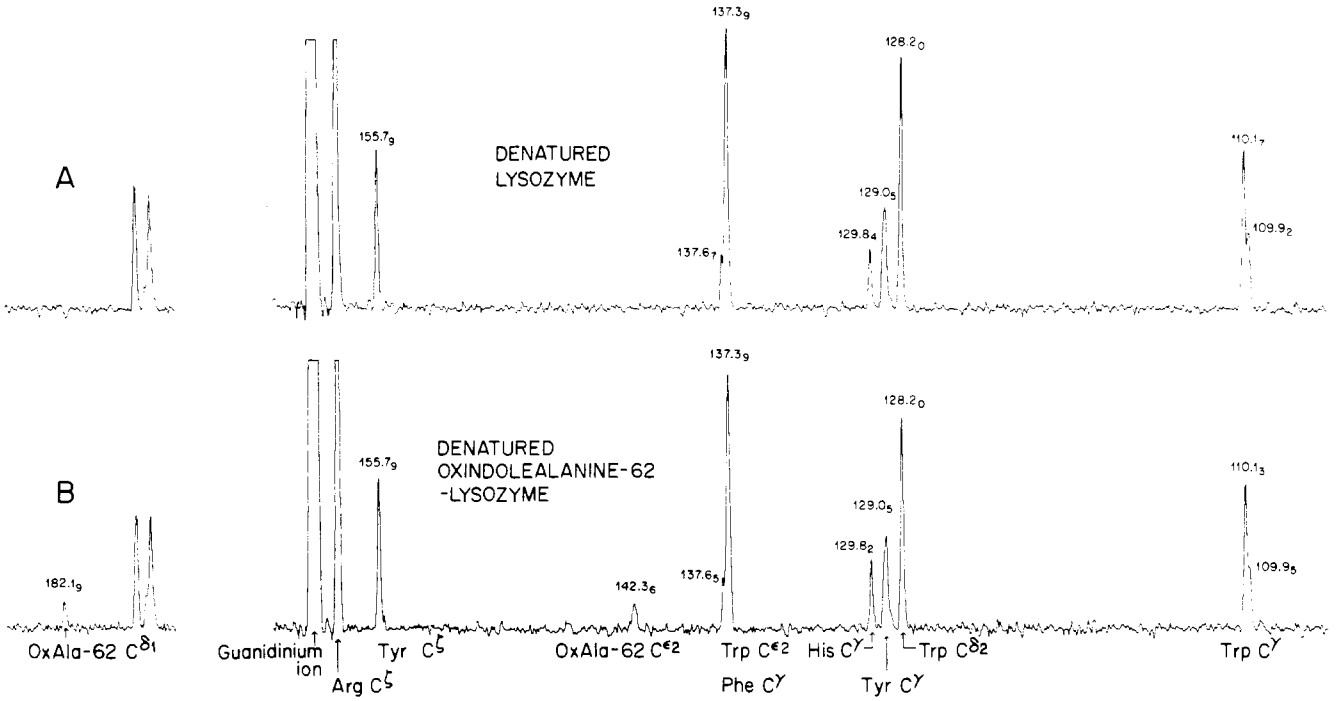

FIGURE 3: Region of aromatic carbons and downfield edge of the carbonyl region in convolution-difference natural-abundance ^{13}C-NMR spectra of denatured hen egg-white lysozyme and [oxindolealanine-62]lysozyme. Each spectrum was recorded under conditions of noise-modulated off-resonance proton decoupling, with 65 536 accumulations and a recycle time of 2.2 s (40 h total time). Numbers above the peaks are chemical shifts in ppm downfield from Me_4Si. Assignments of the resonances of unmodified protein residues are those of Allerhand et al. (1973b). Assignments for oxindolealanine-62 (labeled OxAla-62) are described in the text. (A) 8.5 mM hen egg-white lysozyme and 6.1 M guanidinium chloride in H_2O (0.1 M NaCl, pH 3.0, 50 °C). (B) 8.3 mM [oxindolealanine-62]lysozyme and 6.1 M guanidinium chloride in H_2O (0.1 M NaCl, pH 3.0, 50 °C). The reaction with *N*-bromosuccinimide was carried out in 0.1 M [1-^{13}C]acetate (15% enriched). This sample was prepared from one of the two described in the caption of Figure 4 (after data accumulation for Figure 4C was completed) by dialysis for 3 days against distilled water at 4 °C and addition of guanidinium chloride.

recorded many days after the reaction of the lysozyme sample with *N*-bromosuccinimide. In Figure 4 we show some spectra of the native modified protein recorded at various intervals after completion of the reaction. The times given in Figure 4 (and subsequent figures) are intervals from the transfer of the sample to 25 °C (after completion of the reaction and dialysis at 4 °C for 20 h) to the time of 50% completion of the spectral accumulation. Other details are given in the caption of Figure 4.

A comparison of Figure 4C with 1B indicates that [oxindolealanine-62]lysozyme is the only clearly observable species in Figure 4C. However, the spectrum of Figure 4A, recorded soon after completion of the reaction of lysozyme with *N*-bromosuccinimide, indicates the formation of a species other than oxindolealanine at position 62. In this spectrum, the resonances of C$^{\delta_1}$ (peak a) and of C$^{\epsilon_2}$ (peak b + c) of oxindolealanine-62 each have the intensity of about half a carbon. Furthermore, Figure 4A contains three resonances (peaks x, y, and z) which are not detectable in Figure 4C, and which have approximately half-carbon intensities. We conclude that 18 h after completion of the reaction of lysozyme with *N*-bromosuccinimide only about 50% of the chemically modified residue 62 is oxindolealanine. An examination of Figure 4A–C indicates that the transient species converts slowly into oxindolealanine-62. Peak x of Figure 4A can be seen (but barely) in Figure 1B.

Identification of [δ_1-*Acetoxytryptophan-62*]*Lysozyme.* The facile conversion of the intermediate observed in Figure 4A into an oxindolyl group (Figure 4C) suggests the presence of an intact *o*-phenylene group in the intermediate. Furthermore, peaks x, y, and z (Figure 4A) require the presence of at least three nonprotonated carbons. The chemical shift of peak z (Table I) is consistent with an assignment to C$^{\gamma}$ of a tryptophan derivative that has an oxygen or nitrogen at C$^{\delta_1}$ (see Table 5.47 of Stothers, 1972). These observations strongly suggest that the intermediate is either the enol form of an oxindolyl group (2-hydroxyindolyl group, Figure 2B), or an ester of this enol (Figure 2C). A precedent for the formation of a stable ester of the enol form of an oxindolealanine residue is provided by the oxidation of 2 mol of lysozyme with 1 mol of I_2. The product of this reaction is the internal Glu-35 ester of [δ_1-hydroxytryptophan-108]lysozyme (Imoto and Rupley, 1973; Beddell et al., 1975). The ^{13}C-NMR spectrum of this modified lysozyme (Norton, R. S., and Allerhand, A., unpublished results) yields resonances at 141.1, 131.7, and 95.4 ppm, which are very close to peaks x, y, and z, respectively, of Figure 4A (Table I).

Unlike the case of residue 108, there are no protein carboxyl groups close enough to participate in ester formation with residue 62 (Imoto et al., 1972). Therefore, we investigated the possibility of esterification with the acetate buffer. We used two procedures to show that the moiety under consideration is indeed an ester of acetic acid (δ_1-acetoxytryptophan, Figure 2C). First, we observed the time dependence of the ^{13}C-NMR spectrum of a lysozyme sample that had been treated with *N*-bromosuccinimide in the presence of [1-^{13}C]acetate. Second, we observed the time dependence of the ^{13}C-NMR spectrum of a lysozyme sample that had been treated with *N*-bromosuccinimide in the absence of acetate. The two sets of results are presented in the following two subsections.

We assign peaks x, y, and z to C$^{\epsilon_2}$, C$^{\delta_2}$, and C$^{\gamma}$, respectively, of δ_1-acetoxytryptophan-62. We have not observed a resolved resonance for C$^{\delta_1}$ of this residue. An examination of chemical shifts of model compounds strongly suggests that this reso-

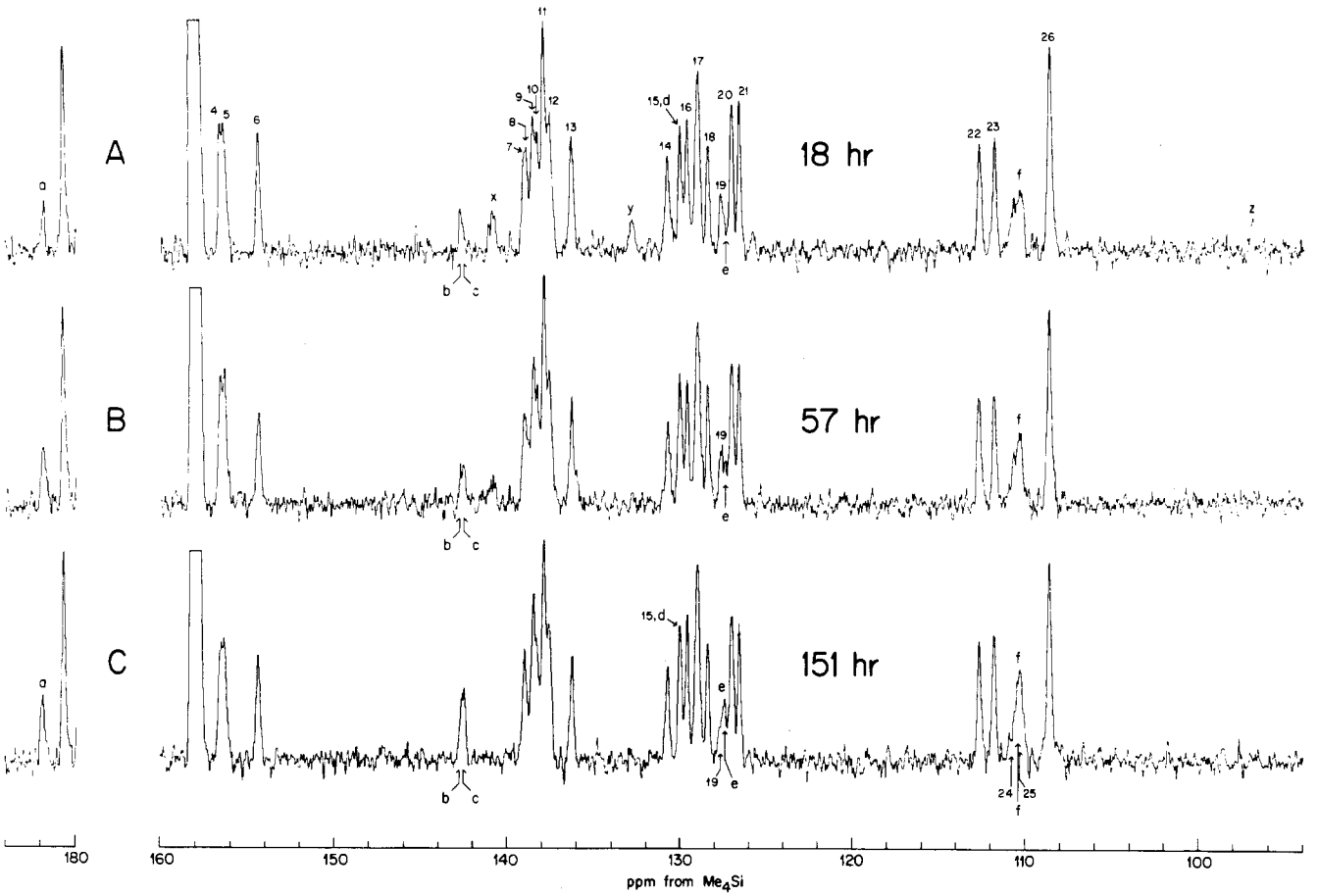

FIGURE 4: Time dependence of the region of aromatic carbons and of the downfield edge of the carbonyl region in convolution-difference natural-abundance ^{13}C NMR spectra of hen egg-white lysozyme treated with an equimolar amount of *N*-bromosuccinimide. Each spectrum is the sum of two spectra obtained from two different samples at similar times after transfer to 25 °C. In both cases, *N*-bromosuccinimide treatment was carried out in 0.1 M acetate buffer (of natural isotopic composition in one case, and 15% ^{13}C-enriched on the carbonyl in the other). In each case, spectra were recorded under conditions of noise-modulated off-resonance proton decoupling, with a recycle time of 2.2 s and 32 768 accumulations (20 h total time) per spectrum. Peak designations are those of Figure 1 and Table I. Arrows labeled b, c, e, f, 19, 24, and 25 indicate chemical shifts of corresponding resonances of Figure 1. Solution conditions for each sample were: 14.7 mM protein in H_2O (pH 3.9, 0.1 M NaCl, 25 °C). The resonance of C^{γ} of His-15 (peak 15) shifts slightly downfield when going from pH 3.1 (Figure 1) to 3.9, and as a result it coincides with peak d at pH 3.9. (A) 18 h after transfer of each sample to 25 °C. (B) 66 and 48 h after transfer to 25 °C of the modified proteins prepared with unenriched and enriched acetate, respectively (average time 57 h). (C) 151 h after transfer of each sample to 25 °C.

nance falls under the large peak of C^{ζ} of the eleven arginine residues, at about 158 ppm (Norton, R. S., and Allerhand, A., unpublished results).

Reaction in the Presence of ^{13}C-Enriched Acetate. Figures 5A–E show the time dependence of the carbonyl region in the spectrum of the modified protein prepared with the use of acetate buffer 90% ^{13}C-enriched on the carbonyl. Figure 5F is the corresponding spectral region of [oxindolealanine-62]-lysozyme prepared with the use of acetate of natural isotopic composition. The significant feature in Figure 5 is the resonance at 172.6_8 ppm in the spectra of the sample prepared with the use of ^{13}C-enriched acetate. The chemical shift of this resonance is similar to reported chemical shifts of carbonyl groups of acetyl esters (Stothers, 1972). The time dependence of the integrated intensity of this resonance (Figure 5A–E, and spectra recorded at 27 and 78 h) yields a half-life of 44 h for the species that gives rise to this resonance, consistent with the intensity changes of peaks x, y, and z in Figure 4. Furthermore, the intensity of this resonance, extrapolated to zero time and corrected for the ^{13}C enrichment and other factors (see below) is similar to the initial intensities of peaks x, y, and z (Figure 4A).

In order to determine the concentration of [δ_1-acetoxytryptophan-62]lysozyme from the intensity of the carbonyl resonance at 172.7 ppm, we first measured the T_1 and NOE of this resonance, because of the likelihood that methyl group rotation significantly affects these parameters. We obtained experimental values of 1.7 s for T_1, and 1.3_6 for the NOE (see Experimental Procedure). The intensity at zero time (taken as the time of transfer of the sample from 4 to 25 °C) was obtained by extrapolation from the time dependent behavior in Figure 5A–D and two analogous spectra recorded at 27 and 78 h. After corrections for differences in NOE, T_1, and isotopic composition, a value of 0.4_8 was obtained for the ratio of the zero-time intensity of the carbonyl resonance to the average single-carbon intensity of the nonprotonated aromatic carbon resonances.

Reaction in the Absence of Acetate. The spectra of the product of the reaction of lysozyme with *N*-bromosuccinimide in the absence of acetate (Figure 6A,B) provide additional evidence for the involvement of acetate in the formation of [δ_1-acetoxytryptophan-62]lysozyme. In contrast to the behavior in the presence of acetate, the intensities of peaks a and b + c in Figure 6A indicate nearly complete conversion of residue 62 into oxindolealanine soon after completion of the reaction in the absence of acetate. δ_1-Acetoxytryptophan cannot be detected.

There appear to be some differences between the spectrum

TABLE I: Chemical Shifts of Some ^{13}C Resonances of [δ_1-Acetoxytryptophan-62]Lysozyme and [Oxindolealanine-62]-Lysozyme.

Assignment		Peak	Chemical
Protein[a]	Carbon[b]	Designation[c]	Shift[d]
B	62-δ_1	a	181.9
A	Acetoxy		172.7
B	62-ϵ_2	b	142.8
		c	142.6
A	62-ϵ_2	x	140.9
A	63-ϵ_2	8[e]	138.9
B	63-ϵ_2	9[f]	138.6
A	62-δ_2	y	132.8
B	62-δ_2	d	130.1
		17[g]	129.0
A	63-δ_2	19[e]	127.7
B	63-δ_2	e	127.5
A	63-γ	24[e,h]	110.7
B	63-γ	f	110.4
A	62-γ	z	97.0

[a] A and B designate [δ_1-acetoxytryptophan-62]lysozyme and [oxindolealanine-62]lysozyme, respectively. [b] 63 Indicates Trp-63, and 62 indicates either δ_1-acetoxytryptophan- or oxindolealanine-62. Greek letters are carbon designations of Figure 2. The term acetoxy indicates the carbonyl of the 2-acetoxyindolyl group. [c] Peak designations are those of Figures 1B, 4, and 6. [d] In ppm downfield from Me_4Si. Estimated accuracy is ±0.1 ppm. Values for [δ_1-acetoxytryptophan-62]lysozyme were measured from the spectrum given in Figure 4A. Values for [oxindolealanine-62]lysozyme were obtained from the spectra in Figures 1B, 4, and 6. [e] Assignment based on time-dependent intensity in Figure 4. [f] Arises from $C^{\epsilon 2}$ of residue 63 of one or both forms of [oxindolealanine-62]lysozyme, and from C^{γ} of a phenylalanine residue (Oldfield et al., 1975b). [g] Two other carbons also contribute. [h] Downfield component of peak f in Figure 4A, with about the same chemical shift as peak 24 of the intact protein.

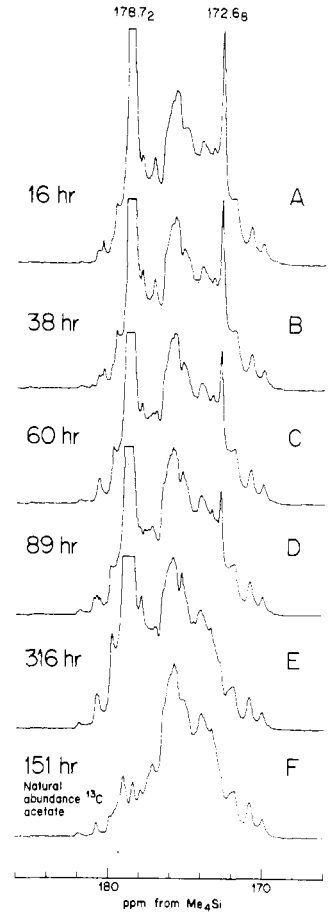

FIGURE 5: (A–E) Time dependence of the carbonyl regions in the ^{13}C NMR spectra of hen egg-white lysozyme treated with an equimolar amount of *N*-bromosuccinimide in the presence of 0.2 M [1-^{13}C]acetate (90% ^{13}C-enriched). Solution conditions were: 13.8 mM protein in H_2O, pH 3.9, 0.1 M NaCl, 25 °C. Times given in each spectrum are intervals after transfer to 25 °C. Spectra were recorded under conditions of noise-modulated off-resonance proton decoupling, and processed with 0.88 Hz digital broadening. For spectra A–D, 32 768 accumulations with a recycle time of 1.1 s (10 h total time per spectrum) were used. For spectrum E, 49 152 accumulations with a recycle time of 2.2 s (30 h total time) were used. The small numbers in spectrum A are chemical shifts in ppm downfield from Me_4Si. The strong peak at 178.7$_2$ ppm arises from the carbonyl of $CH_3{}^{13}COOH$ (in fast exchange with acetate), which is present as a result of incomplete dialysis, and also arises from hydrolysis of [δ_1-acetoxytryptophan-62]lysozyme. (F) Same as spectra A–E, except that 0.1 M acetate of natural isotopic composition was present during treatment with *N*-bromosuccinimide, protein concentration was 14.8 mM, and 32 768 accumulations with a recycle time of 2.2 s (20 h total time) were used.

of [oxindolealanine-62]lysozyme shown in Figure 6A and spectra of this and other samples of [oxindolealanine-62]lysozyme recorded several days after completion of the reaction (Figures 6B,C and 4C). Increases in the intensities of peaks c and d with time suggest that initially the amount of one of the two forms of [oxindolealanine-62]lysozyme is lower than that of the other, and that several days are required for equilibrium to be established at room temperature.

Minor Components. There are features in our spectra that suggest the presence of species other than [δ_1-acetoxytryptophan-62]lysozyme and [oxindolealanine-62]lysozyme in the product formed under the reaction conditions of Hayashi et al. (1965). For example, the measured intensity of peak x (see Figure 4A,B) is consistently greater than the corresponding intensities of peaks y and z. Furthermore, the initial spectrum of the modified protein prepared in the absence of acetate (Figure 6A) contains a weak broad resonance at the position of peak x, but no detectable resonances at the positions of peaks y and z. The broad resonance disappears when going to Figure 6B. These observations suggest that [δ_1-acetoxytryptophan-62]lysozyme is not the only unstable modified lysozyme that contributes to peak x.

Peak f of the modified protein (Figures 1B, 4, and 6), which was assigned above to C^{γ} of Trp-63, yields a measured intensity that varies from one sample to another, but is consistently greater than expected for one carbon. The intensities of peaks a and b + c of Figures 1B, 4C, and 6C suggest that there is only 80–90% conversion of Trp-62 into oxindolealanine. Therefore, C^{γ} of intact Trp-62 may yield a contribution of up to 0.2 carbon to the intensity of peak f. However, the intensity of peak f is greater than 1.2 in most spectra. Additional contributions to peak f may arise from brominated tyrosine residues, since it is known that *N*-bromosuccinimide can brominate tyrosine residues at C^{ϵ} (Witkop, 1961; Mathur and Narang, 1975). The brominated carbons of 3-bromo-4-hydroxytoluene and 3,5-dibromo-4-hydroxytoluene have chemical shifts of 110.3 and 111.1 ppm, respectively (for 1 M solutions in dioxane at 28 °C). The chemical shift of peak f of the modified protein is about 110.4 ppm (Table I).

Concluding Remarks

A solution of lysozyme that has been treated with *N*-bromosuccinimide as prescribed by Hayashi et al. (1965) contains time-dependent amounts of [δ_1-acetoxytryptophan-62]lyso-

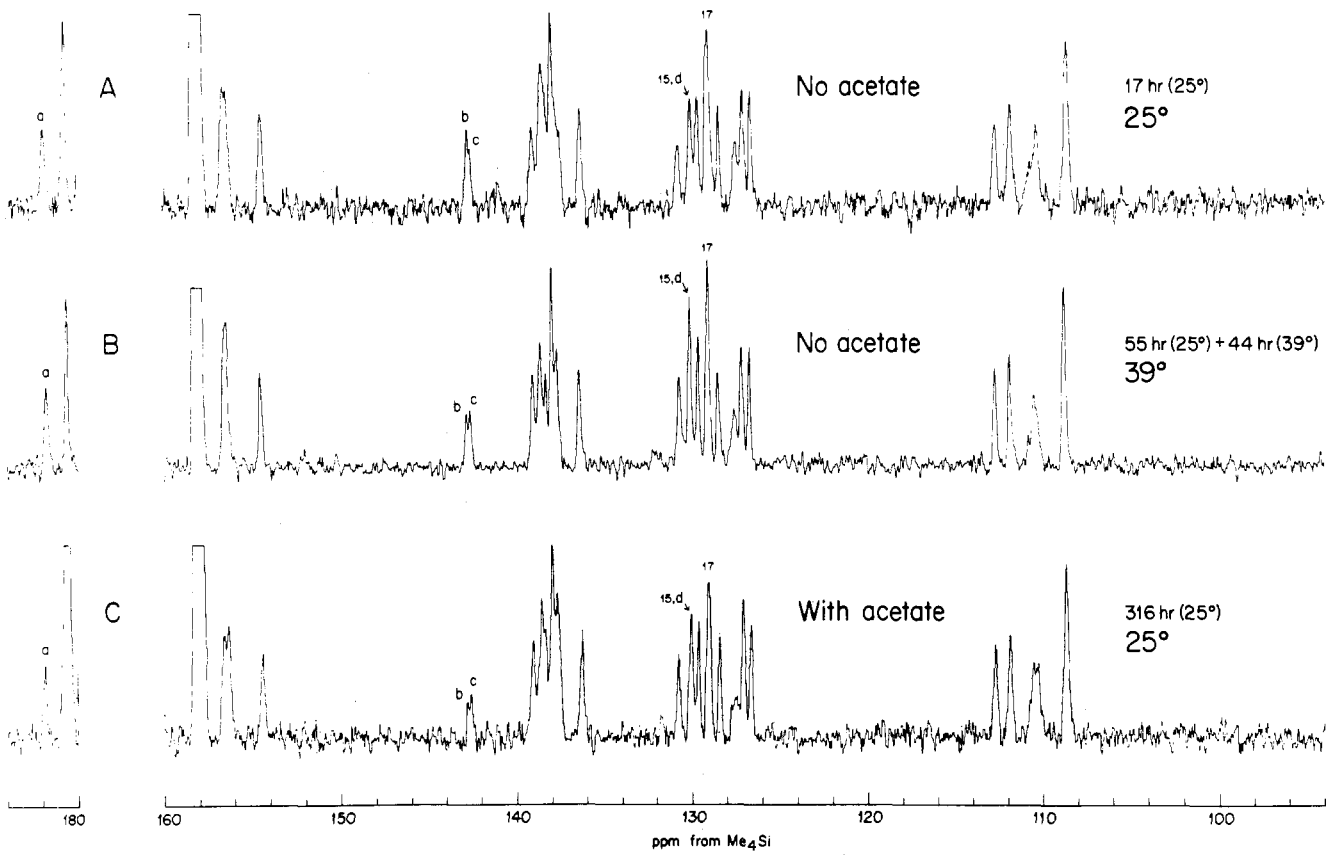

FIGURE 6: Region of aromatic carbons and downfield edge of the carbonyl region in convolution-difference natural-abundance ^{13}C-NMR spectra of hen egg-white lysozyme treated with an equimolar amount of *N*-bromosuccinimide in the absence (A, B) and in the presence (C) of acetate buffer. Peak designations are those of Figure 1 and Table I. Each spectrum was recorded under conditions of noise-modulated off-resonance proton decoupling, with 49 152 accumulations and a recycle time of 2.2 s (30 h total time). (A) Lysozyme treated with *N*-bromosuccinimide in the absence of acetate buffer (16.5 mM protein in H_2O, pH 3.9, 0.1 M NaCl, 25 °C), 17 h after transfer to 25 °C. (B) As spectrum A, but recorded at 39 °C and 99 h after transfer to 25 °C (44 h after transfer to 39 °C). (C) Lysozyme treated with *N*-bromosuccinimide in the presence of 0.2 M [1-^{13}C]acetate (90% enriched). The time-domain data is the same as that of Figure 5E.

zyme and of two forms (probably the two diastereoisomers) of [oxindolealanine-62]lysozyme, in addition to minor protein components. Our results indicate that if the procedure of Hayashi et al. (1965) is followed exactly, then after dialysis the predominant species are the two forms of [oxindolealanine-62]lysozyme. Recently, Cooper (1974) replaced the 4-day dialysis step of Hayashi et al. (1965) by desalting on a Sephadex G-25 column. This modified procedure may yield a protein that contains a higher initial percentage of [δ_1-acetoxytryptophan-62]lysozyme than the protein obtained by the method of Hayashi et al. (1965).

Finally, our results have implications for the use of *N*-bromosuccinimide for modifying tryptophan residues of proteins in general. The reaction has often been carried out in acetate or formate buffer (Spande and Witkop, 1967a-c) and, therefore, it is possible that esters of δ_1-hydroxytryptophan have been formed. If oxindolealanine derivatives are desired, steps should be taken to ensure the absence of such esters. Our results also suggest the possibility of studying the esters as chemically modified species in their own right. Furthermore, it may be possible to use the *N*-bromosuccinimide reaction to attach reporter groups at C^{δ_1} of some tryptophan residues of proteins, by carrying out the reaction in the presence of interesting potential adducts.

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Dental Phosphoprotein-Induced Formation of Hydroxylapatite during in Vitro Synthesis of Amorphous Calcium Phosphate†

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ABSTRACT: (Ethylenedinitrilo)tetraacetic acid soluble phosphoproteins were isolated from rat incisor and bovine unerupted teeth. This material was examined for its effect on the stability of amorphous calcium phosphate in vitro. When the precipitation of amorphous calcium phosphate was attempted in the presence of small amounts of these phosphoproteins, an apatite-like mineral was observed to form, which was approximately 60% crystalline, as determined by infrared measurements. This apatite phase could not be induced by addition of phosphoprotein after the precipitation reaction. The organic phosphate bound to these phosphoproteins was shown to be directly responsible for the formation of the apatite phase, since removal of 60% of the covalently bound phosphate with alkaline phosphatase destroyed the protein's ability to induce hydroxylapatite formation. The properties of the dental phosphoproteins appear to be consistent with their possible involvement in the development of the mineral phase of dentine.

The mineral component of bone and teeth has been shown to consist of two phases, a hydroxylapatite-like crystalline phase and an amorphous calcium phosphate phase (Harper and Posner, 1966; Termine and Posner, 1966). Moreover, the crystalline phase increases with age from a value of 30% in femurs of 8-day-old rats to 65% in 26-day-old rats (Termine and Posner, 1966; Posner, 1973). It has been suggested that the earliest mineral to be deposited in bone and dentine matrices is the amorphous fraction (Termine and Posner, 1967). With maturation, the amorphous content of these tissues is reduced to a limiting value of about 10%, although a somewhat disordered carbonate containing analogue of hydroxylapatite may also be present (Posner and Betts, 1975). In order to characterize the amorphous component of bone mineral, extensive investigations have been carried out on synthetic ACP[1] (Posner and Beebe, 1975; Posner and Betts, 1975). The ACP formed under basic conditions is believed to be similar to that which occurs biologically (Betts et al., 1975; Posner and Betts, 1975).

The organic components of bone and dentine matrices may play an important role in the stabilization or modification of the mineral phase. A number of laboratories have reported the occurrence of phosphoproteins in bones and teeth (for a review see Leaver et al., 1975). The nature of the phosphoprotein fraction isolated appears to depend on the type of extraction used, that is, whether acid or neutral, degradative or nondegradative (Dickson et al., 1975). However, one characteristic of this protein fraction that is consistently observed is its high content of aspartic acid (>30% residue) and serine (>30% residue, some of which is *O*-phosphoserine). Recently, experiments by Weinstock and Leblond (1973) demonstrated the rapid appearance of serine and phosphate at the mineralization front of the rat incisor, strongly supporting the early suggestion by Veis and Perry (1967) that dentine phosphoprotein is involved in mineralization. The studies reported here were undertaken to further define the role that dentine phosphoprotein may play in mineralization and, specifically, to determine its effects on the formation of hydroxylapatite.

Experimental Procedure

Unerupted bovine teeth were obtained from the lower jaws of cattle between 1 and 2 years of age. All operations were carried out at 4 °C. After removal from the dental sacs, the teeth were fractured, freed of pulp, and repeatedly washed with cold 0.45 M NaCl. A 24-h soak in 7 M guanidine hydrochloride (pH 7) removed the last remnants of soft tissue. After thorough washing with distilled water, the teeth were extracted with 1.5 volumes of 0.35 M EDTA (pH 8.1) four times over a period of 11 days. The combined EDTA extracts were dialyzed against distilled water until free of calcium and lyophilized.

Incisors were taken from 130–140 g male Wistar rats. After removing the apex, the tooth was freed of pulp and washed

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[1] Abbreviations used are: ACP, amorphous calcium phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; NBS, National Bureau of Standards.