# Phosphorylation Modulates Keratin Structure†

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ABSTRACT: Bovine hoof keratin was shown to be a substrate for cAMP-dependent protein kinase using [$\gamma$-$^{32}$P]ATP. Natural-abundance cross-polarization (CP) MAS $^{13}$C NMR was used to examine the effect of phosphorylation on keratin structure. When short contact times were used, phosphorylation was shown to increase the number of residues in the motionally restricted portions of the protein; i.e., a portion(s) of the protein became more rigid upon phosphorylation. Circular dichroism (CD) spectra showed a spectral shape characteristic of $\alpha$ helix for this keratin. Phosphorylation of the keratin by cAMP-dependent protein kinase resulted in a CD spectrum with the same shape but of greater apparent intensity. This may have been the result of an increase in the $\alpha$-helical content of the protein. These data showed that the structure of keratin changed significantly upon phosphorylation by cAMP-dependent protein kinase. The region of the keratin molecule most likely to be altering its structure was the end of the molecule, which was involved in the formation of, and intracellular attachment of, intermediate filaments. Therefore, these data suggested that cAMP-dependent phosphorylation may produce significant changes in the intracellular organization of intermediate filaments. When the keratin was phosphorylated using cold ATP, magic-angle spinning (MAS) $^{31}$P nuclear magnetic resonance (NMR) revealed two resonances arising from the phosphorylation sites on the keratin. The more shielded resonance was shown to arise from cAMP-dependent protein kinase phosphorylation. Static $^{31}$P NMR measurements suggested that at least two classes of cAMP-dependent sites existed with the same isotropic $^{31}$P chemical shift; one was considerably motionally restricted with respect to the other.

Intermediate filaments (IF)[1] and their phosphorylation have been the subject of considerable recent investigation. Studies several years ago demonstrated that IF are phosphoproteins. Cell-free phosphorylation of desmin demonstrated that this type of IF is a substrate for cAMP-dependent kinase (Gibbs et al., 1985; O'Conner et al., 1979, 1981). Studies by Gard and Lazarides (1982a,b) further showed that cAMP-dependent phosphorylation of desmin occurs in vivo as well. Gilmartin et al. (1980) described cAMP-dependent phosphorylation of keratins in cultured cells. More recently, Inagaki et al. (1987, 1988) and Evans (1988) clearly demonstrated that in vitro phosphorylation of vimentin and desmin resulted in disassembly of filaments. Other work (Ando et al., 1989; Evans, 1988) localized the phosphorylation sites in the N-terminal region of the desmin polypeptide. A correlation between phosphorylation state and IF organization during mitosis also was documented (Chou et al., 1989; Evans, 1984; Evans & Fink, 1982; Fey et al., 1983). Similar phosphorylation-induced disassembly was shown for nuclear lamins (Dessev & Goldman, 1988; Georgatos et al., 1988; Worman et al., 1988). Conversely, neurofilaments became increasingly phosphorylated as they were transported down the axon, and this phosphorylation was accompanied by increased stability (Nixon et al., 1989). In the case of keratin IF, phosphorylation did not induce disassembly (Eckert & Yeagle, 1988).

We have recently shown that IF organization in cultured PtK1 cells was dramatically affected when cells were exposed to 5 mM acrylamide (Eckert, 1985, 1986). Coincident with acrylamide-induced reorganization of keratin filaments was a significant dephosphorylation of keratin polypeptide (Eckert & Yeagle, 1988). However, little was known concerning specific effects of phosphorylation on the structure of IF which caused the observed changes in assembly and/or organization.

Since the maintenance of keratin IF morphology appeared to be promoted by phosphorylation of keratin (Eckert & Yeagle, 1988), it would be expected that IF morphology was based on the structure of the keratin protein. These studies were designed to determine whether phosphorylation by cAMP-dependent protein kinase altered keratin structure. Approaches were used that were sensitive to both the properties of the phosphorylation sites and the structure of the protein. Cross-polarization (CP) magic-angle spinning (MAS) $^{13}$C nuclear magnetic resonance (NMR) and circular dichroism (CD) were used to study the effects of phosphorylation on the structure of keratin. MAS $^{31}$P NMR was used to study the phosphorylation sites directly. Bovine hoof keratin was used in these initial studies because of the large amount of material required for the NMR experiments.

## Materials and Methods

*Reagents.* cAMP-dependent protein kinase, alkaline phosphatase, and ATP were purchased from Sigma Chemical Co. [$\gamma$-$^{32}$P]ATP was obtained from ICN Radiochemicals (specific activity = 25 Ci/mmol). Ultrapure urea was obtained from Schwarz/Mann. Acrylamide and other electrophoresis reagents were purchased from Bio-Rad.

*Preparation of Keratin.* Bovine keratin was prepared from hoof according to the method of Steinert (1975). Briefly, slices of stratum spinosum from the bed of the hoof were cut into

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[1] Abbreviations: CP, cross-polarization; CSA, chemical shift anisotropy; IF, intermediate filament(s); MAS, magic-angle spinning; NMR, nuclear magnetic resonance.

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small pieces, and the keratin was extracted for 3 h at 37 °C in 8 M urea, 10 mM Tris-HCl, and 1 mM β-mercaptoethanol, pH 9.0. The extract was centrifuged for 1 h at 45000*g*, and the supernatant was further clarified by centrifugation for 1 h at 150000*g*. The extract was dialyzed against 10 mM Tris-HCl and 1 mM β-mercaptoethanol, pH 7.2, to remove urea. The resulting assembled keratin was collected by centrifugation for 30 min at 20000*g*. Approximately 150 mg was obtained from 10 g of hoof material.

*In Vitro Phosphorylation/Dephosphorylation.* Keratin was incubated in buffer P (30 mM NaCl, 25 mM Tris-HCl, and 1 mM $MgCl_2$, pH 7.0) containing 100 μCi of [γ-$^{32}$P]ATP (final ATP concentration was 200 μM) with 5 μg/mL cAMP-dependent protein kinase and 5 μM cAMP for 30 min at 37 °C. Keratin was dephosphorylated by incubation in 1 mg/mL alkaline phosphatase in 50 mM sodium bicarbonate buffer, pH 10.0, for 30 min at 37 °C. After treatment, all samples were dialyzed into 50 mM HEPES (pH 7) and lyophilized. Samples were rehydrated immediately prior to NMR measurements. For NMR studies, nonradioactive ATP was used but other conditions were identical.

*SDS Gel Electrophoresis.* Samples were analyzed by Mini-Slab (Idea Scientific) SDS–polyacrylamide gel electrophoresis on 12.5% gels which were stained with Coomassie Blue. Gels were dried and autoradiograms prepared on Kodak Ortho M X-ray film.

*Assays.* Phosphate content was determined as inorganic phosphate by the method of Bartlett (1959). Protein was determined by the method of Lowry et al. (1951).

*MAS $^{31}P$ NMR.* MAS $^{31}$P NMR spectra were obtained on a Nicolet NT-150 NMR spectrometer using home-built MAS probes to observe $^{31}$P at 60 MHz. Spinning rates of 3000–4000 rps was used. All spectra were collected with $^{1}$H decoupling of $\gamma B_1/2\pi = 30$ kHz. A total of 2048 data points were collected in the time domain and zero-filled to 4096 data points, prior to Fourier transformation. All data were collected in blocks of about 0.5 h each initially. Each block was then checked for any systematic changes in the resulting spectra before adding all the blocks for maximum signal-to-noise. Other spectral details are given in the figure legends. Chemical shifts are expressed relative to external 85% phosphoric acid, using a reference from MAS $^{31}$P NMR of (−)-(2*S*,3*S*)-bis-(diphenylphosphino)butane ("chiraphos").

*CPMAS $^{13}C$ NMR.* The CP (cross-polarization) $^{13}$C NMR measurements were obtained on a Nicolet NT-150 spectrometer at a carbon frequency of 37.735 MHz with a home-built magic-angle spinning unit. Contact times of 0.2, 0.5, and 1 ms were used. The $^{1}$H decoupling field was $\gamma B_1/2\pi = 45$ kHz. The spinner system is a modified version of Wind et al. (1983), with a sample volume of 0.3 mL. The samples were spun at 3200 rps. 4K data points were collected with a spectral width of 20 kHz and an acquisition time of 104 ms. Chemical shifts are relative to external tetramethylsilane, using the signal at 1.2 ppm from the silicone rubber plug as a secondary standard. The keratin was introduced into a Teflon sleeve, and a silicone rubber plug was inserted into the sleeve, which was then pressed into the Kel-F rotor body. Nonviscous air-sensitive liquids have been successfully spun with this arrangement with no liquid leaking out and no air leaking in. The rotor was spun with dry nitrogen gas which had passed through a heat-exchange coil immersed in an ice bath. The sample was maintained at 20 °C.

*Circular Dichroism Spectra.* Circular dichroism (CD) spectra were obtained on a JASCO J-200 spectropolarimeter in 1-mm path-length cells at room temperature. Three spectra were obtained of each sample and then added to improve signal-to-noise of the resulting spectrum. Solutions contained approximately 1 mg/mL protein for the measurements.

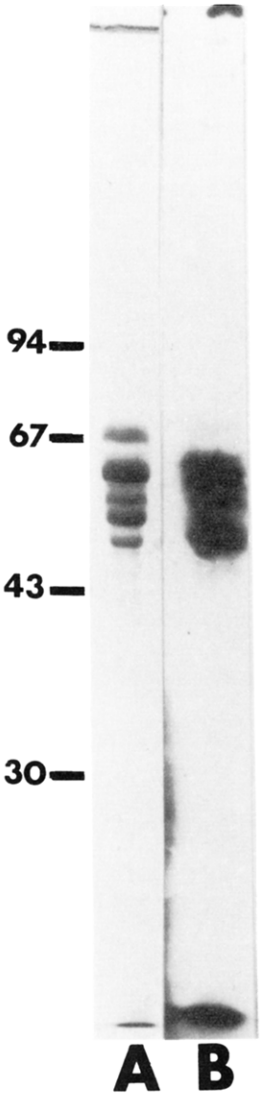


FIGURE 1: SDS–polyacrylamide gel electrophoresis on 12.5% gels of the bovine hoof keratin preparation used in this study and its phosphorylation with $^{32}$P induced by cAMP-dependent protein kinase and [γ-$^{32}$P]ATP, as described in the text. (A) Coomassie Blue stained gel. (B) Autoradiogram of the same gel.

## Results

*Phosphorylation of Bovine Hoof Keratin by cAMP-Dependent Protein Kinase.* Keratin that was isolated and subsequently phosphorylated with cAMP-dependent protein kinase and [γ-$^{32}$P]ATP, as described above, was analyzed on SDS gel electrophoresis. As can be seen in Figure 1, five keratin bands were present in this preparation [verified by Western blots (data not shown)]. $^{32}$P was incorporated by cAMP-dependent protein kinase into four keratin bands present in this preparation: 63, 59, 55, and 51 kDa. A fifth keratin subunit, at 67 kDa, incorporated small amounts of $^{32}$P and was detected only as a faint band. The specific activity of the phosphorylation varied from band to band. Variations of specific activity among keratins have been documented previously (Steinert et al., 1982) and are likely to be due to differences in the phosphoserine content which may reflect differences in the number of phosphorylation sites among keratins (Steinert et al., 1982; Steinert, 1988). These data showed that this hoof keratin was a substrate for cAMP-de-

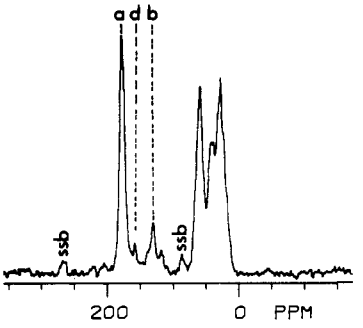


FIGURE 2: CPMAS $^{13}C$ NMR spectrum at 37.735 MHz of phosphorylated keratin (treated first with phosphatase and subsequently with cAMP-dependent protein kinase, as described in the text). 50000 transients were obtained using a 0.5-ms contact time and a 1-s repetition rate.

pendent protein kinase, as was the keratin of the PtK1 cells we have studied previously (Eckert and Yeagle, manuscript in preparation).

In vitro phosphorylation of hoof keratin did not result in significant disassembly as it did for vimentin and desmin (Evans, 1988; Inagaki et al., 1987, 1988). Rather, in vivo dephosphorylation of keratin accompanied reorganization of IF (Eckert & Yeagle, 1988).

*Effect of Phosphorylation on Keratin Structure. (A) CPMAS $^{13}C$ NMR.* Although phosphorylation of keratin has been observed previously, no information on the effects of phosphorylation on the structure of the keratin polypeptide has as yet been reported. $^{13}C$ NMR in general provides a powerful means to study protein structure. However, in a rodlike molecule like keratin, only limited molecular motion is possible. Many of the $^{13}C$ NMR resonances of the keratin would be expected to be broadened by expression of the $^{13}C$ chemical shift anisotropy. Although ordinary, liquid-state $^{13}C$ NMR techniques are feasible with keratin (Mack et al., 1988), they would reveal clearly only resonances from the more mobile regions of the polypeptide. Other carbons would yield resonances too broad to be observed by this approach.

CPMAS $^{13}C$ NMR allows one to obtain information about relatively immobile regions of the keratin molecule. MAS introduces the scaling factor $(3 \cos^2 \theta - 1)$ into the terms describing the line width of the observed resonances. Therefore, MAS collapses the resonances from all the observable phosphates in the sample to their isotropic resonances when the sample is rapidly spun at the angle that reduces the aforementioned scaling term toward zero, or the "magic angle" of 54.7°. Cross-polarization depends upon the static component of the $\{^1H-^{13}C\}$ dipolar term of the Hamiltonian, which in turn is largest at long correlation times (i.e., slow motions). Therefore, the $^{13}C$ NMR resonances that are favored in the CP experiment are those that have nearby protons and have motions characterized by long correlation times, i.e., regions of the molecule that are relatively immobile. Cross-polarization occurs during a "mixing time", or "contact time", a period in which the nuclear spin system of the carbons is deliberately brought into thermal contact with the spin system of the protons (Pines et al., 1973). In general, the shorter the "contact time", the stronger the static component of the $\{^1H-^{13}C\}$ dipolar interaction has to be to effectively cross-polarize. Therefore, resonances that appear at short "contact times" correspond to relatively immobile carbons.

CPMAS $^{13}C$ NMR spectra were obtained of phosphorylated (by cAMP-dependent protein kinase after stripping off phosphate with phosphatase) keratin. As a result of the preparation protocol, cAMP-independent phosphorylation sites

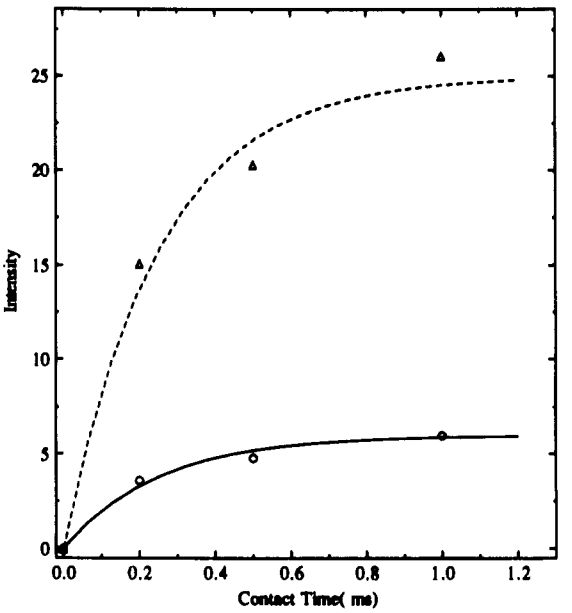


FIGURE 3: $^{13}C$ NMR resonance intensity from CPMAS spectra as a function of contact time for dephosphorylated keratin. (Δ) Carbonyl resonance; (O) major aromatic envelope. The lines drawn represent a $T_{C-H}$ of 0.25 ms. However, the accessible signal-to-noise and number of data points in these experiments did not permit an accurate determination of $T_{C-H}$.

were not phosphorylated and therefore did not contribute to the properties of the protein as reflected in these data. A representative example is given in Figure 2. A contact time of 0.5 ms was used, which is a relatively short contact time (a similar spectrum is observed at 0.2-ms contact time, but of lower intensity). Therefore, the spectrum in Figure 2 selected for the more immobile regions of the keratin molecule. The spectrum contained resonances arising from the aliphatic carbons (more shielded envelope of resonances), from the aromatic carbons (in the middle of the spectrum, b), and also from the carbonyls (the furthest deshielded, a). The latter resonances gave rise to spinning side bands, because of the larger $^{13}C$ chemical shift tensors exhibited by the carbonyls than by the aliphatic carbons. No spinning side bands were observed from the aromatic resonances because of the low relative resonance intensity.

Figure 3 shows the effect of changing the mixing time, or contact time, in the CP experiment on dephosphorylated keratin (phosphate removed by phosphatase treatment). The rapid rise of resonance intensity for the carbonyl and aromatic resonances at short contact time characterizes $T_{C-H}$, the cross-polarization time constant. The limited signal-to-noise available has precluded a full characterization of the CP spin dynamics, but the limited data set did allow for an estimate of $T_{C-H}$, neglecting the effect of rotating-frame spin–lattice relaxation, $T_{1\rho}$, which dominates the long mixing time behavior. These data suggested a similar $T_{C-H}$ for both the aromatic envelope and the carbonyl envelope. The relatively short values of $T_{C-H}$ suggested by these data indicated strong dipolar coupling, arising in part from motions characterized by relatively long correlation times.

The aliphatic resonances exhibited a decreasing intensity with increasing contact time over the range measured in these experiments (data not shown). These data suggested that the rise in resonance intensity, characteristic of $T_{C-H}$, occurred prior to the first sampling at a 0.2-ms contact time. What was observed was likely the decay due primarily to $T_{1\rho}$ of the protons. Such a short $T_{C-H}$ relative to the carbonyls, for example, could be expected since the aliphatics have directly

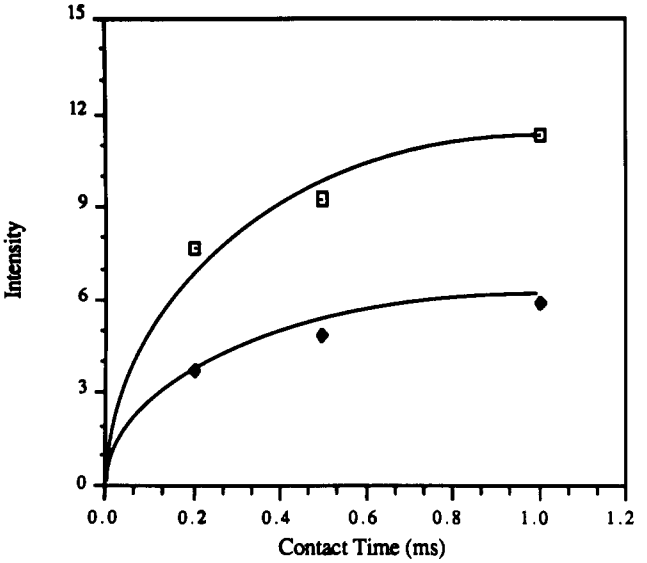


FIGURE 4: Differential effect of phosphorylation on CPMAS $^{13}C$ NMR resonance intensity of the major aromatic resonance envelope as a function of contact time. In these experiments, the resonance intensity of the carbonyls was nearly identical in the phosphorylated and dephosphorylated samples at each contact time, thus providing an internal control. (⊡) Phosphorylated keratin (treated first with phosphatase and subsequently with cAMP-dependent protein kinase, as described in the text); (♦) dephosphorylated keratin.

bonded protons leading to particularly strong dipolar interactions.

The CPMAS $^{13}C$ NMR spectra were then measured for phosphorylated keratin as a function of contact time. Qualitatively similar results were obtained for the phosphorylated keratin as for the dephosphorylation keratin. However, a quantitative difference was noted (Figure 4). The resonance intensity of the aromatic envelope was larger at all contact times for the phosphorylated keratin than for the dephosphorylated keratin. Consequently, at all contact times, a larger percentage of the total protein aromatic carbons resonating in this region of the keratin spectrum contributed to the CPMAS $^{13}C$ NMR spectrum of the phosphorylated keratin than of the dephosphorylated keratin. Apparently phosphorylation caused an immobilization of a portion of the keratin polypeptide rich in aromatic amino acids. Such an immobilization would have increased the percentage of the aromatic amino acid carbon atoms with strong dipolar coupling to protein protons and thus increased the contribution of those carbons to the CPMAS spectrum. (Alternatively, phosphorylation might have suppressed intensity from the carbonyl and aliphatic groups in the CPMAS experiment; absolute spin counting was not feasible in these experiments.)

Therefore, the CPMAS $^{13}C$ NMR data suggested that the response of the keratin polypeptide to phosphorylation was, at least in part, a conformational change. We then exploited another means to detect protein conformational changes, circular dichroism, to see if supportive evidence could be found for a conformational change in the keratin heteropolymer due to phosphorylation.

*(B) Circular Dichroism Measurements.* Circular dichroism (CD) spectra were obtained of phosphorylated (by cAMP-dependent protein kinase after stripping off phosphate with phosphatase) and dephosphorylated (by phosphatase) keratin. The results are shown in Figure 5. CD spectra in the far-UV region, as shown here, reflect the secondary structure of the protein. Optical transitions ($n \leftrightarrow \pi^*$) diagnostic for $\alpha$ helix and $\beta$ sheet are observed in the 200–230-nm region. The spectra obtained here are characteristic of $\alpha$ helix as the

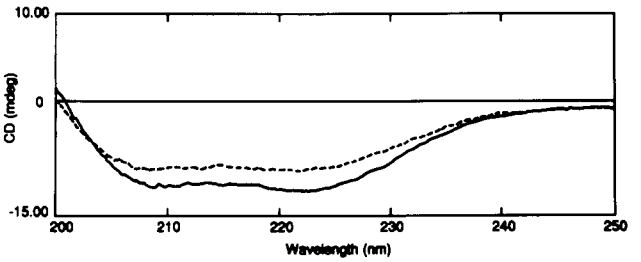


FIGURE 5: Circular dichroism spectra of phosphorylated (treated first with phosphatase and subsequently with cAMP-dependent protein kinase, as described in the text) (—) and dephosphorylated bovine hoof keratin (treated with phosphatase) (---). Spectra were obtained in 1-mm path-length cells at a concentration of 1 mg/mL keratin.

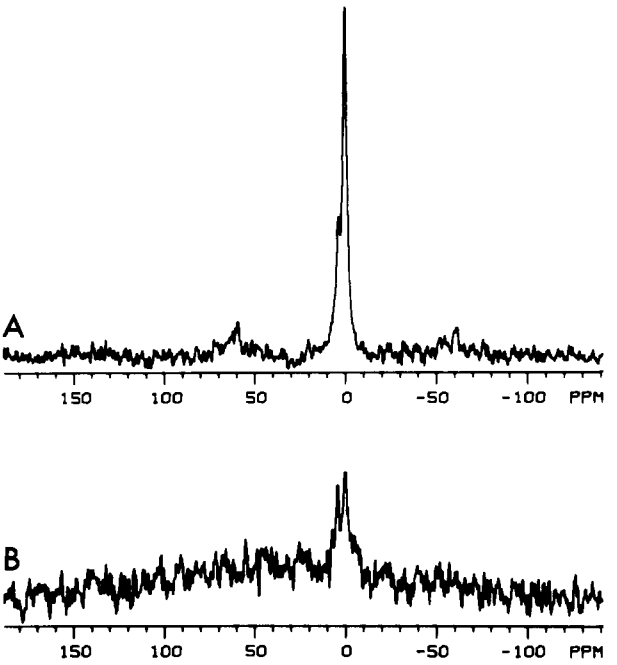


FIGURE 6: FT MAS $^{31}P$ NMR at 60 MHz of bovine hoof keratin. 40000 transients were obtained with 4K data points and a 2-s repetition rate, transformed with a line broadening of 30 Hz. A spinning rate of about 3600 rps was used. (A) Keratin phosphorylated with cAMP-dependent protein kinase and [$^{31}P$]ATP as described in the text. The phosphorylation level was 0.1 $\mu$mol of phosphate/mg of protein. (B) Keratin with basal phosphorylation. The spectrum in (A) showed a greatly enhanced resonance intensity for the more shielded $^{31}P$ NMR resonance induced by the cAMP-dependent protein kinase phosphorylation. The less shielded resonance was still apparent, but only as a very small shoulder on the major resonance, suggesting that it was not enhanced significantly (or certainly not proportionately) by cAMP-dependent protein kinase phosphorylation.

preponderant secondary structure (troughs at 209 and 222 nm). This result is consistent with the suggested coiled-coil of $\alpha$ helices for the rod portion of the keratin molecule (Fraser et al., 1964).

Comparison of the phosphorylated keratin with the dephosphorylated keratin revealed no detectable difference in the shape of the CD transitions. However, there was a difference in the magnitude of the transitions. In general, the transitions were more intense for the phosphorylated protein. This change may be supportive evidence for a conformational change upon phosphorylation. One possible explanation of this result was a phosphorylation-induced increase in the amount of polypeptide involved in $\alpha$ helix.

*Observation of Phosphorylation Sites on Keratin with MAS $^{31}P$ NMR.* Keratin that was isolated and phosphorylated with cAMP-dependent protein kinase, as described above, but with [$^{31}P$]ATP, was examined with MAS $^{31}P$ NMR. The result is shown in Figure 6A. Two $^{31}P$ resonances are observed, one

much more intense than the other. The observation of differing chemical shifts suggests significantly different chemical environments for the phosphorus at these phosphorylation sites.

The sample used in Figure 6A had been washed by centrifugation prior to NMR analysis, and all inorganic phosphate, cAMP, and ATP was removed. The absence of these phosphate-containing compounds is confirmed by the absence of $^{31}P$ NMR resonances corresponding to these compounds in the spectra obtained (such resonances would appear in the region −5 to about −20 ppm). Keratin that was stripped of phosphate by phosphatase and washed by centrifugation showed no $^{31}P$ resonance (data not shown). Therefore, the observed $^{31}P$ NMR resonances in the samples of phosphorylated keratin most likely arise from the phosphorylation sites on the keratin.

The two resonances in Figure 6A were then further assigned by comparison to two other preparations. Figure 6B shows untreated keratin, with only a low, basal level phosphorylation. Comparison of Figure 6A and Figure 6B shows that the more shielded resonance is greatly enhanced by cAMP-dependent phosphorylation.

Further confirmation of the assignment was achieved by preparing keratin with only one kind of phosphorylation. Keratin was stripped of all phosphate by phosphatase treatment. The effectiveness of stripping was verified by phosphate and protein analysis of the final preparation. This protein was then rephosphorylated by cAMP-dependent protein kinase. The spectrum of this preparation showed only the more shielded resonance of Figure 6A (data not shown). We therefore concluded that the more shielded resonance arises from cAMP-dependent protein kinase phosphorylation and the less shielded resonance (Figure 6A) arises from other kinase activity.

Figure 6A revealed additional information about the cAMP-dependent protein kinase phosphorylation sites. Two spinning sidebands were observed, centered around the cAMP-dependent protein kinase induced resonance. These spinning sidebands appeared because the MAS rate did not satisfy the relationship $\nu_{rot} \gg \Delta\nu_{CSA}$, required if all the resonance intensity is to be incorporated in the centerband of the spectrum [where $\nu_{rot}$ = the spinning rate and $\Delta\nu_{CSA}$ = the frequency range of the $^{31}P$ chemical shift anisotropy (CSA) powder pattern that was to be averaged by the MAS]. This observation indicates that at least some of the phosphorylation sites gave rise to a $^{31}P$ powder pattern of substantial breadth. Therefore, only limited motional narrowing was experienced at those sites that contributed to the (relatively) broad $^{31}P$ powder pattern.

To better understand this phenomenon, we studied a model compound, polycrystalline phosphoserine, since many of the phosphorylation sites on keratin have been suggested to be serines, with a smaller number of threonines (Steinert, 1988). The MAS $^{31}P$ NMR result is shown in Figure 7A. In addition to the strong centerband, two spinning sidebands were observed. The $^{31}P$ CSA powder pattern was revealed by the nonspinning experiment (Figure 3B). The principle elements of the chemical shift tensor ($\sigma_{11}$, $\sigma_{22}$, and $\sigma_{33}$) are evident and agree with published results (Seelig, 1978).

To test the suggestion further in the phosphorylated keratin that the spinning sidebands reflect the presence of a $^{31}P$ powder pattern, nonspinning $^{31}P$ NMR spectra were obtained of a keratin sample that had been first stripped by phosphatase and then rephosphorylated with cAMP-dependent protein kinase. The result of this difficult experiment is shown in Figure 8. The maximum amount of protein that could be fit into the sample spinner was employed to obtain this spectrum. The

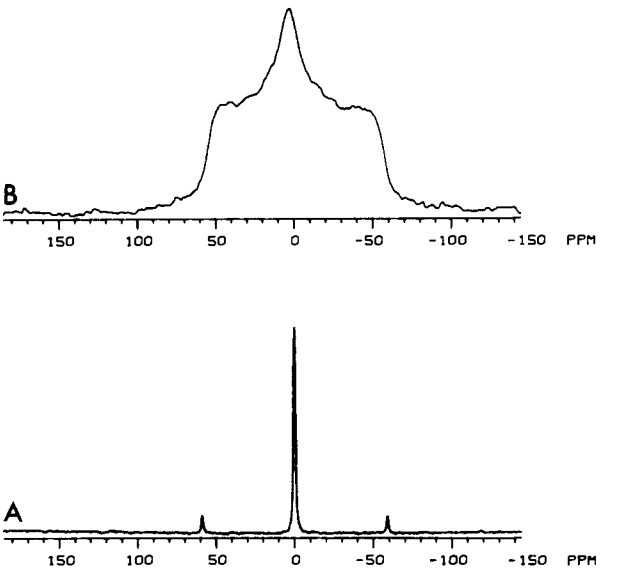


FIGURE 7: (A, lower panel) FT MAS $^{31}P$ NMR spectrum at 60 MHz of a powder of polycrystalline phosphoserine. (B, upper panel) FT $^{31}P$ NMR spectrum at 60 MHz of a powder of phosphoserine. 264 transients were collected with a repetition rate of 10 s. The transform was calculated by using an expontential filter of 120 Hz. Approximately 0.5 $cm^3$ of dry powder was used in the spinner for these experiments, and 4K data points were collected.

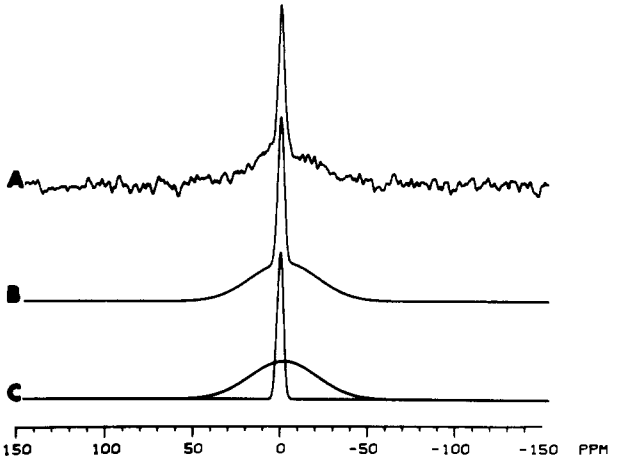


FIGURE 8: (A) FT $^{31}P$ NMR spectrum at 60 MHz of phosphorylated keratin (treated first with phosphatase and subsequently with cAMP-dependent protein kinase, as described in the text). 40 000 transients were obtained and 4K data points with a 2-s repetition rate, transformed with a line broadening of 100 Hz. The phosphorylation level was 0.3 μmol of phosphate/mg of keratin. (B) Computer-generated deconvolution of the spectrum in (A) into two components, using a Gaussian approximation. The resulting line shape supports the interpretation of the spectrum in (A) as arising from two components, the simplest (though not a unique) explanation of the observed data. This deconvolution does not presuppose any particular attributes to the line shape of the broad resonance in (A) and therefore should not be interpreted in terms that relative the line shape to the details of the molecular motion involved in the averaging of the chemical shift tensor. (C) The two separate components used to create (B). The broad resonance accounts for approximately three-fourths of the total intensity in this analysis.

signal-to-noise for this spectrum could not be as good as that for the model compound, phosphoserine, because of the much higher concentration of phosphate in the latter. What could be readily seen in this spectrum (Figure 8) was a relatively broad powder pattern. In addition, a more narrow feature was also included in the spectrum. Although this could be a single powder pattern reflecting, perhaps, slow, multiple site reorientation, the simplest explanation was the presence of a broad powder pattern with a relatively narrow resonance superim-

posed. The spectral simulation showed how the spectrum could be deconvoluted.

If the deconvolution was correct, these results suggested the presence of (at least) two kinds of cAMP-dependent protein kinase phosphorylation sites. One kind of site was relatively immobile, giving rise to the broad $^{31}P$ powder pattern (and producing spinning sidebands in the MAS $^{31}P$ experiment). The broad powder pattern in the $^{31}P$ NMR spectrum of the phosphorylated protein was much narrower than that observed from the immobile polycrystalline phosphoserine, however, consistent with substantial large-amplitude, rapid (>10 kHz) motion for these phosphorylation sites on the keratin. Therefore, the sites reflected in the $^{31}P$ NMR spectra were not strictly immobilized; they were motionally restricted in comparison to the other sites that apparently gave rise to the narrow $^{31}P$ NMR resonances. The relatively immobile sites were likely sites on a portion of the protein without much conformational flexibility. Most of the motional narrowing likely arose from the axial diffusion around the phosphate–serine link, with some limited additional contributions from local diffusion around the bonds of the serine methylene to the $\alpha$-carbon and to the hydroxyl.

The other kind of site was relatively mobile, giving rise to a much more narrow resonance (even in the non-MAS experiment) due to rapid and disordered motion of the phosphate at these sites. The most likely source for such extensive motional narrowing would be substantial conformational flexibility of the polypeptide chain in the regions of these phosphorylation sites, in addition to the contributions from the motions described above. However, some inhomogeneity in the state of aggregation of the keratin polypeptides that might also contribute to the observation of a heterogeneous $^{31}P$ NMR resonance cannot be ruled out.

## Discussion

The experiments reported here employed NMR to probe for structural changes induced in keratin by phosphorylation by cAMP-dependent protein kinase. Hoof was used as a source for these natural-abundance NMR studies because of the large amount of protein required for these experiments. We decided to use whole keratin filaments, which were heteropolymers, rather than individual purified keratin subunits. It is clear from Figure 1 that the keratin subunits have different specific activities, probably due to different numbers of phosphorylation sites. The phosphorylation sites, however, are on the same portion of all of the keratin subunits (Steinert & Roop, 1988). These variations in phosphorylation sites may impart slight differences in properties to each keratin polypeptide. Although the spectra observed contained contributions from all five different subunits and represent the sum of their properties, use of the heteropolymer enabled us to examine structural changes of keratin in native filaments. This approach appeared to be valid at this level of discrimination, since all keratins (types I and II) have fundamentally similar structures (Steinert & Roop, 1988) with phosphorylation sites on the amino and carboxyl terminals of the keratin polypeptides.

The CPMAS $^{13}C$ NMR experiments showed a significant structural change upon phosphorylation. These data suggested that a portion of the molecule became relatively rigid that had not been rigid previously.

An important question to answer was what portion of the protein is becoming more rigid upon phosphorylation. The keratin family of proteins exhibits considerable sequence homology. Particularly strong homology is found in the central regions of the polypeptide that are felt to constitute a rodlike region of the protein (Steinert & Roop, 1988). Sequence analysis indicates that the regions of repeating heptads that comprise much of the rod region are poor in aromatic amino acids. Only the fifth and seventh positions of the repeating heptad contain hydrophobic amino acids, and most of them are not aromatic (Crewther et al., 1978). The absence of bulky amino acids allows the relatively close contact between adjacent helices required to form the coiled coil. The carboxyl- and amino-terminal regions of the keratin were generally more rich in aromatic amino acids (Crewther et al., 1978). Furthermore, the NMR studies of Mack et al. (1987, 1988) showed that the rod region of the keratin molecule was already quite rigid. These data then suggested that the most likely source for the phosphorylation-induced changes in the aromatic amino residues was in a structural alteration in the end, variable regions of the keratin.

Data from other laboratories demonstrated that phosphorylation of keratin occurs on the amino and carboxyl terminals of the polypeptide chains (Steinert, 1988). Collectively, those data plus the results in the present study indicated that an alteration in conformation (perhaps a change in the mobility) of the ends of the keratin molecule occurred upon cAMP-dependent phosphorylation.

This is a region of the keratin molecule proposed to be involved in the formation of the IF and in particular in the formation of intracellular attachment sites (Albers & Fuchs, 1989). Structural changes in this region of the protein would be expected to produce changes in the distribution of IF since it would affect contact sites between keratin units making up the intermediate filament, as well as interactions between keratin filaments and other cellular components. An increase in the extent of relatively rigid $\alpha$ helix in the keratin could lead to a more rigid rodlike molecule that would be more likely involved in an extended and perhaps more rigid morphology rather than a collapsed morphology [as was observed for dephosphorylated keratin in acrylamide-treated cells (Eckert & Yeagle, 1988)]. However, these suggestions are presently speculation since the data presented here cannot be used to unambiguously assign the regions of the molecule contributing to the CPMAS $^{13}C$ NMR spectrum at short contact times or causing the changes in the CD. Therefore, while these data strongly suggest a structural change in the IF, we cannot say at this time definitely which part of the molecule or which subunits are responsible for the structural change.

The $^{31}P$ NMR studies revealed the presence of two distinct phosphorylation sites. These phosphorylation sites were characterized by slightly different isotropic $^{31}P$ chemical shifts. The more shielded resonance appears to be the result of cAMP-dependent phosphorylation. The small difference in chemical shifts between the two kinds of sites indicates a small difference in chemical environment of each kind of phosphorylation site and is likely caused by the differences in the amino acid sequences characteristic of each class of phosphorylation site. Interestingly, the lack of chemical shift dispersion for the cAMP-dependent protein kinase phosphorylation sites indicates that the chemical structure around the majority of these sites is the same. That result is consistent with the specificity of the kinase for substrate.

The cAMP-dependent protein kinase phosphorylation sites can be further subdivided into at least two classes. Although the MAS $^{31}P$ NMR studies showed that all these sites had very similar chemical shifts, the static $^{31}P$ NMR experiments indicated that some of the sites were strongly motionally restricted compared to the other sites. This motional restriction likely derives from the protein structure itself. Some of the

phosphorylation sites must be on rigid regions of the molecule, while others are on relatively mobile regions of the molecule. Since the $^{13}C$ NMR experiments indicated that significant portions of the keratin polypeptide became relatively immobile upon phosphorylation, some of the phosphorylation sites might also be expected to be relatively immobile. The motionally restricted phosphorylation sites were likely on a portion of the variable (with respect to sequence homology among keratins) end regions of the keratin which was rigid prior to phosphorylation or became rigid upon phosphorylation.

## Summary

These data suggest that phosphorylation alters keratin conformation. Thus, it is possible that cAMP-dependent phosphorylation may produce changes in the intracellular organization of keratin intermediate filaments.

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