Characterization of Chemical and Enzymatic Acid-Labile Phosphorylation of Histone H4 Using Phosphorus-31 Nuclear Magnetic Resonance†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) is used to investigate acid-labile phosphorylation of histone H4. ^{31}P NMR detects phosphorylated histidine residues in in vitro enzymatically phosphorylated H4. The source of kinase is nuclei from either regenerating rat liver or Walker-256 carcinosarcoma. When regenerating rat liver is the source, ^{31}P NMR spectroscopy on the denatured phosphorylated protein exhibits a resonance at 5.3 ppm relative to an 85% orthophosphoric acid external reference. This peak corresponds well with the chemical shift of standard π-phosphohistidine scanned under similar conditions. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis confirms acid lability. When the source of kinase is Walker-256 carcinosarcoma, the ^{31}P NMR spectrum contains a resonance at 4.9 ppm which corresponds well with standard τ-phosphohistidine run under the same conditions. Chemical phosphorylation of H4 has been accomplished by using dipotassium phosphoramidate which specifically phosphorylated the imidazole moiety of histidine at neutral pH. $NaDodSO_4$–polyacrylamide gel electrophoresis confirms acid lability, and high-pressure liquid chromatography of protein hydrolysates yields phosphohistidine. ^{31}P NMR of chemically phosphorylated H4 in a structured state reveals two peaks at 4.8 and 7.3 ppm with line widths of 9 and 55 Hz, respectively. These resonances indicate that both histidine residues of H4 (His-18 and His-75) are phosphorylated, the latter relatively immobile and the former relatively free in solution. ^{31}P NMR studies on chemically phosphorylated peptide fragments of H4, namely, H4(1–23) and H4(38–102), confirm this model of H4 structure.

Histones are closely associated with deoxyribonucleic acid (DNA)[1] in nucleosomal particles. Correlation of postsynthetic modifications of histones with functional and structural aspects of chromatin is currently being intensively investigated to elucidate the mechanisms involved in the control of transcription and cell replication [for review, see Isenberg (1979)]. One postsynthetic modification of histones studied in this context is phosphorylation of hydroxy amino acid residues (Arfmann & Bode, 1980; Fischer & Laemmli, 1980; Gurley et al., 1978; Langan, 1978; Szopa et al., 1980). The occurrence of phosphorylation on basic amino acid residues of histones has also been described (Chen et al., 1974, 1977; Smith et al., 1973, 1974). The phosphorylation of basic amino acids has the distinct properties of being acid labile and relatively base stable (Smith et al., 1978). Early studies by other groups have shown that acid-labile phosphorylation (Schiltz & Sekeris, 1969) and phosphohistidine (Zetterqvist & Engstrom, 1966) exist in proteins from rat liver nuclei. It has been demonstrated that in rapidly growing cells, such as those from regenerating rat liver and Walker-256 carcinosarcoma, lysine residues of histone H1 and the histidine residues of histone H4 are phosphorylated (Chen et al., 1974). Such modifications have been usually overlooked since procedures of isolating and characterizing histones generally involve acid conditions at one point or another.

Phosphorus nuclear magnetic resonance[1] (^{31}P NMR) has been used to detect and characterize phosphate derivatives of amino acids in proteins. NMR is also a powerful probe to macromolecular structure. The binding sites of proteins have been studied by using small molecules containing NMR-detectable nuclei as intermolecular probes [for example, see Dower & Dwek (1979) and Schnackerz et al. (1979)]. Changes in chemical shifts and relaxation times can be detected by NMR upon association of protein with the probe which can be a substrate or modifier. Properties of the binding site can therefore be obtained in this manner. For example, binding sites of mouse myeloma immunoglobulin and adenylate kinase have been characterized by ^{31}P NMR using phosphorylcholine (Goetze & Richards, 1977) and adenosine triphosphate (Rao et al., 1979), respectively, as intermolecular probes. Intramolecular probes (probes covalently attached to the protein), both physiological and nonphysiological, have been used to study protein conformation. For example, by utilization of ^{1}H NMR, protons of aromatic residues such as those on histidine have been used as physiological probes to the structure of superoxide dismutases (Burger et al., 1980). Trifluoroacetylated derivatives of lysine have been used as nonphysiological probes to study the conformation of lysozyme [for review, see Sykes & Hull (1978)] by employing ^{19}F NMR.

Since phosphorylation is prevalent among proteins and since it has an important role in the regulation of these proteins, covalently bound phosphate may serve as physiological intramolecular probes to protein conformation. ^{31}P NMR is used here to detect phosphohistidine on phosphorylated H4 and to demonstrate that these phosphoramidates can be used as intramolecular probes to H4 structure.

Materials and Methods

Materials. [γ-^{31}P]ATP and [^{32}P]orthophosphoric acid were purchased from ICN Pharmaceuticals. $POCl_3$ was obtained from Mallinckrodt, Inc., and was distilled. Calf thymus whole

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[1] Abbreviations used: DNA, deoxyribonucleic acid; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; ppm, parts per million; peptide H4(1–23), a peptide of histone H4 containing residues 1–23 inclusive; D_2O, deuterium oxide.

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histones, ATP, and $NaDodSO_4$ (95%) were purchased from Sigma Chemical Co. D_2O (99.8%) was obtained from Bio-Rad Laboratories. Chemicals used for $NaDodSO_4$–polyacrylamide gels were of ultrapure grade. All other chemicals were of reagent grade. Chromex DA-X12-11 anion-exchange resin was obtained from Durrum Chemical Co. Histone H4 was purified from calf thymus whole histones by the method of Bohm et al. (1973). Chymotryptic fragment H4(38–102) was obtained by the procedure of Crane-Robinson et al. (1977). Peptide fragment H4(1–23) was prepared by a dilute acetic acid hydrolysis technique as described by Lewis et al. (1975). [^{32}P]$POCl_3$ was synthesized by an exchange method involving [^{32}P]orthophosphoric acid and $POCl_3$, described by O'Brien (1960) as modified by Smith & Theisen (1967). Dipotassium phosphoramidate was synthesized by the method of Stokes (1893). Dipotassium [^{32}P]phosphoramidate was synthesized from [^{32}P]$POCl_3$ by the method of Stokes (1893) as modified by Smith & Theisen (1967).

Methods. Chemical phosphorylation of H4 was accomplished by dissolving histone H4 and phosphoramidate in a 1:1 weight ratio in water. After incubation overnight at room temperature, the sample was dialyzed against several changes of water to remove unreacted phosphoramidate.

Partial hepatectomies were done on ether anesthetized Sprague-Dawley rats (200–300 g) according to the method of Higgins & Anderson (1931). Approximately 40–60% of the liver was removed, and 18–19 h were allowed for regeneration.

Isolation of nuclei was accomplished by two methods. Nuclei from Walker-256 carcinosarcoma were obtained by essentially the method of Chauveau et al. (1956). The nuclear fraction from regenerating rat liver was obtained by the method of Lin et al. (1976). Regenerating rat liver was homogenized in 2.2 M sucrose, 15 mM $MgCl_2$, 0.25 mM spermine, 1 mM PMSF, and 1 mM NaF at 7 mL/g of wet weight, then passed through six to eight layers of cheesecloth, and then placed on a 2.2 M sucrose shelf. The crude nuclear pellet was obtained by centrifugation at 40000*g* for 70 min with a SW 27 rotor.

Enzymatic phosphorylation of H4 for polyacrylamide gel electrophoresis was done in 0.1- or 0.2-mL volumes containing 50 mM glycine buffer, pH 9.5, 10 mM $MgCl_2$, 60–200 μM [γ-^{32}P]ATP (2×10^{10} cpm/mL), and 50–100 μg of H4 and started with addition of nuclei (100–200 μg of protein). Incubation was done at 37 °C for 5–10 min and was stopped with either a neutral or an acid quench. A 2× solution of neutral quench contained 4% $NaDodSO_4$, 20% glycerol, 10% 2-mercaptoethanol, and 0.25 M Tris-HCl, pH 7–8. A 2× solution of acid quench contained 4% $NaDodSO_4$, 20% glycerol, 10% 2-mercaptoethanol, and 0.6–1.0 N HCl. Enzymatic phosphorylation of H4 for ^{31}P NMR was done in 40-mL volumes containing 40–80 mg of H4 with the same concentration of buffer, $MgCl_2$, and ATP. The reaction was started by the addition of nuclei. Incubation was carried out a 37 °C, and after 5 min an additional amount of ATP (to the final concentration) was mixed. After another 5 min of incubation, the mixture was quenched with 4 mL of 10% $NaDodSO_4$, dialyzed against several changes of 0.1% $NaDodSO_4$, pH 8 ($NaHCO_3$), and lyophilized. For NMR analysis, the sample was dissolved in 4–5 mL of a D_2O/H_2O mixture.

Polyacrylamide gel electrophoresis was essentially done according to the method of Laemmli (1970) as modified by Weintraub et al. (1975). Resolving gels (10 or 25 cm long) were 15% polyacrylamide with an acrylamide/bis(acrylamide) ratio of 300:4 in 0.1% $NaDodSO_4$ and 0.375 M Tris-HCl, pH 8.8. The stacking gels (1 cm) were 6% polyacrylamide with an acrylamide/bis(acrylamide) ratio of 300:8 in 0.1% $NaDodSO_4$ and 0.125 M Tris-HCl, pH 8.0. Gels were run at 150 mV with a running buffer consisting of 0.38 M glycine, 0.05 M Tris-HCl, pH 8.8, and 0.1% $NaDodSO_4$. Gels were stained in 0.1% Coomassie Blue and 25% 2-propanol made alkaline with $NaHCO_3$ (pH 8) and were destained in 10% 2-propanol adjusted to pH 8 with $NaHCO_3$. Autoradiography was done on Du Pont Chronex 4 X-ray film.

High-pressure liquid chromatography (HPLC) sample preparation and analysis were done by a modification of the method of Steiner et al. (1980). Protein samples were hydrolyzed in 3 N KOH at 120 °C in sealed ampules. Samples were neutralized by addition of 2 N $HClO_4$, and insoluble salts were removed by centrifugation. After lyophilization, the sample was dissolved in H_2O, and any insoluble material was removed by centrifugation. About 100 μL of the hydrolysate was injected into the HPLC system. Elution was done on a 2 × 250 mm column of Chromex DA-X12-11 anion-exchange resin at room temperature with 60 mM potassium phosphate, pH 6.9, at a flow rate of 1 mL/min. Fractions of 1 mL were collected, and radioactivity was measured by Cherenkov counting. Amino acids were *O*-phthalaldehyde derivatized and detected by fluorometry. Standard phospho amino acids were obtained as described by Steiner et al. (1980).

Determination of acid lability by dialysis techniques was done by two methods. (1) A neutral solution of ^{32}P-labeled H4 was placed in a dialysis membrane tubing and was allowed to equilibrate with the surrounding neutral solution. The pH of the surrounding solution was then adjusted to 11 with NaOH, and the ^{32}P content of the surrounding solution was monitored by counting samples of the solution, on a liquid scintillation counter. The pH was then adjusted to 1 with HCl, and the ^{32}P content of the surrounding solution was monitored as before. (2) Phosphorylated proteins run on neutral $NaDodSO_4$ polyacrylamide gels were tested for acid lability by the following microdialysis technique: A piece of dialysis membrane (Spectrapor 3) was used to cover a plastic scintillation vial (Wheaton 986644) containing 0.5 mL of 0.2 N HCl. A gel slice was placed on the membrane along with 1 drop of water. The membrane was secured in place by screwing a cap onto the vial. A cavity was formed which contained the gel slice. The capped vial was then inverted and agitated for several hours. The vial was next reverted, the cap along with the membrane was removed, and the vial was recapped and counted by Cherenkov counting. The control involved dialysis against water instead of acid.

^{31}P NMR spectra were obtained on a Bruker WP-200 NMR spectrometer operated in the Fourier transform mode at 81.02 MHz for ^{31}P resonance and equipped with a field-frequency lock on the deuterium resonance. The sample tube diameter was 10 mm. Sample volumes were 2–5 mL with at least 20% D_2O. Data collected were not proton decoupled. Typical parameters used were 3-kHz spectrometer width, 70° pulse angle, 166-μs dwell time, 8000 data points in free induction decay, 1.0-s relaxation delay. pH was measured before and after scanning and was uncorrected for deuterium isotope effect. The reference peak shown on all spectra is the resonance of 85% orthophosphoric acid contained in a coaxial capillary tube. Chemical shifts were corrected with 0 ppm positioned at the corrected value for 85% orthophosphoric acid. The sample temperature was 300 ± 2 K. Usually 10000–20000 transients were taken.

Results

Phosphorylation by Regenerating Rat Liver Nuclear H4

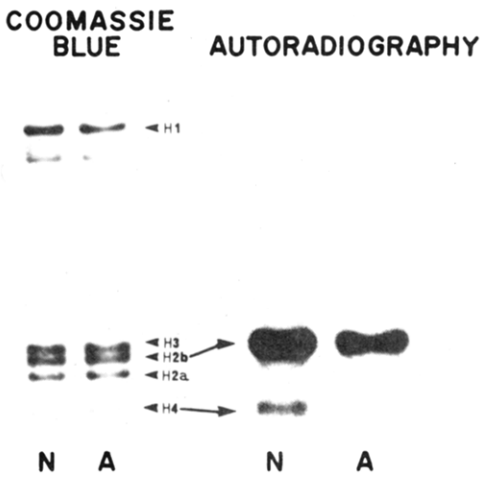

FIGURE 1: Phosphorylation of histones by crude nuclear preparation of regenerating rat liver. Samples were treated with neutral quench, N, or acid quench, A, and run on a neutral $NaDodSO_4$–polyacrylamide gel as described under Materials and Methods. The photograph of the autoradiogram was done on a larger scale.

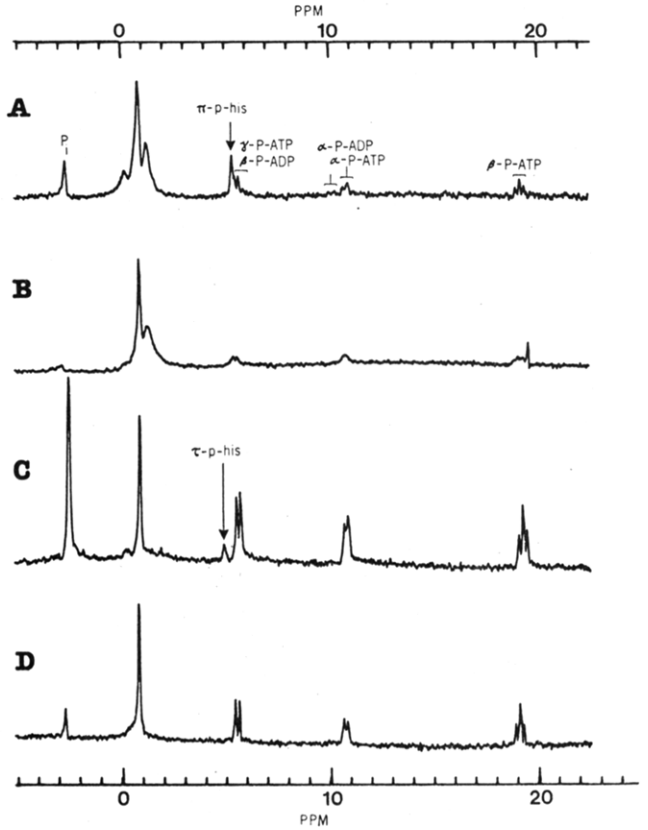

FIGURE 2: ^{31}P NMR of in vitro enzymatically phosphorylated histone H4. The reaction was done by using crude nuclear preparations from 19-h regenerating rat liver, A, or Walker-256 carcinosarcoma, C, as the source of kinase under the conditions outlined under Materials and Methods. Spectra B and D are controls (no added histone H4) to A and C, respectively. The number of transients is 17 000. Note that samples contain $NaDodSO_4$ and have a pH of 10.

Kinase. The $NaDodSO_4$–polyacrylamide gel and corresponding autoradiogram (Figure 1) indicate the presence of acid-labile phosphorylation in H4 as well as some in histone H2B. No phosphorylation of histone is detected by autoradiography when the reaction mixture is quenched with $NaDodSO_4$ prior to addition of the enzyme source (data not shown), thus ruling out any possibility of nonspecific binding in $NaDodSO_4$ gels of labeled nucleotide to the highly basic histones.

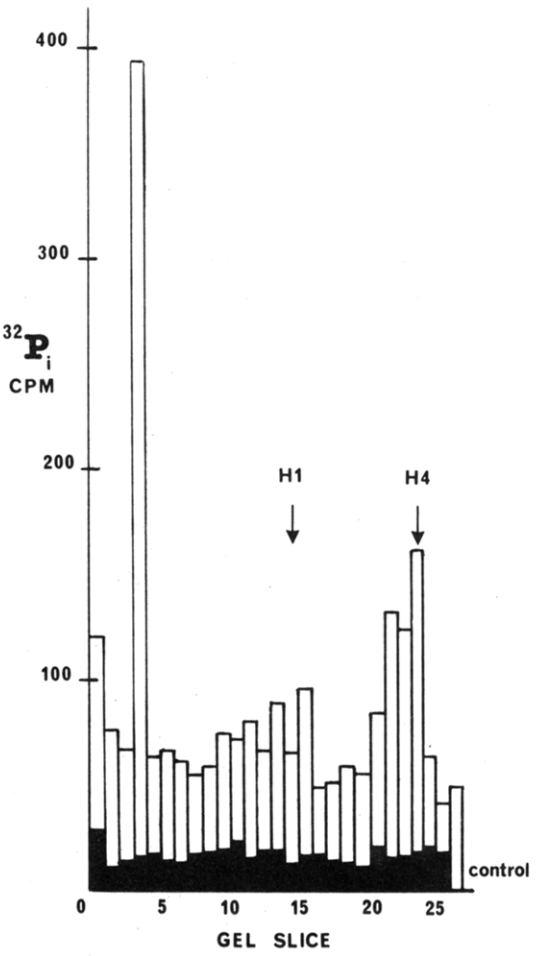

FIGURE 3: Acid-labile phosphorylation of histones by crude nuclear preparation of Walker-256 carcinosarcoma. In vitro enzymatic reaction and neutral $NaDodSO_4$–polyacrylamide gel electrophoresis were done as described under Materials and Methods. Gels (10-cm resolving gel) were sliced (3-mm portions) and assayed for acid lability by the dialysis technique described under Materials and Methods.

The ^{31}P NMR spectrum of the $NaDodSO_4$-quenched mixture of the H4 kinase reaction with added exogenous H4 shows a number of peaks (Figure 2A). Peaks at −3.8, 1.3, 5.8, 10.9, and 19.8 ppm have been assigned to inorganic phosphate, DNA, γ-phosphate of ATP, α-phosphate of ATP, and β-phosphate of ATP, respectively. The locations of the α-phosphate of ADP and β-phosphate of ADP resonances were determined by running standard ADP under similar conditions. The resonance at 5.32 ppm correlates well with π-phosphohistidine standard at pH 10. The peak at 0.8 ppm is a reference peak, and the peak at 0.2 ppm is unassigned but may be lipid phosphates. The control (no added histone H4) (Figure 2B) shows no π-phosphohistidine and very little inorganic phosphate in contrast. The control indicates that resonances contributed by phosphorylation on other proteins in the NMR sample to be sufficiently low in concentration to go undetected under the conditions used. The absence of phosphohydroxy amino acid resonances is in agreement with earlier studies (Bruegger, 1977). Due to the general lability of the phosphoramidate linkages, phosphate to protein ratios were not determined.

Phosphorylation by Walker-256 Carcinosarcoma. Acid lability of phosphorylation by Walker-256 carcinosarcoma nuclear preparation was demonstrated in a microdialysis technique which measures the total amount of phosphate released after acid treatment instead of the amount of decreased phosphorylation as in the case of autoradiography (Figure 3).

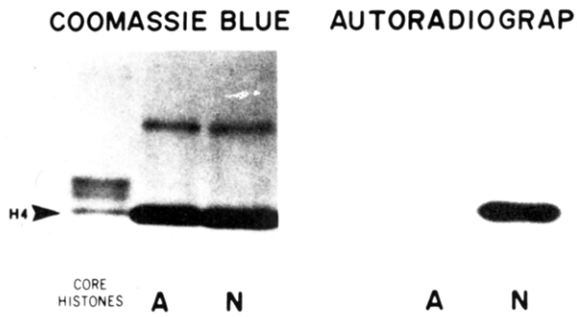

FIGURE 4: Acid lability demonstration of chemically phosphorylated histone H4 on $NaDodSO_4$–polyacrylamide gels. Histone H4 (10 mg) and dipotassium [^{32}P]phosphoramidate (10 mg) were dissolved in 1 mL of H_2O. The solution was allowed to sit overnight at room temperature and was subsequently dialyzed against several changes of H_2O. After dialysis, 40 μL of the phosphorylated H4 mixture was added to 40 μL of a solution containing 4% $NaDodSO_4$, 20% glycerol, 10% 2-mercaptoethanol, and 0.25 M Tris-HCl, pH 8 (neutral loading buffer, N), and another 40 μL of the reaction mixture was added to 40 μL of 4% $NaDodSO_4$, 20% glycerol, 10% 2-mercaptoethanol, and 1 N HCl (acid loading buffer, A). After an incubation of 10 min at 37 °C, the acid-treated samples was neutralized, and 30 μL of each was applied to a neutral $NaDodSO_4$–15% polyacrylamide gel. Standard core histones are shown on the left. Gels were stained in 0.1% Coomassie Blue in 25% 2-propanol adjusted to pH 8 with $NaHCO_3$ and destained in 10% 2-propanol adjusted to pH 8 with $NaHCO_3$. Gels were dried on Whatman 3 MM paper, and autoradiography was done with the aid of intensifying screens. The decrease of H4 phosphorylation in acid-treated samples is clearly shown. The slower migrating protein band in the reaction mixtures is a contaminant.

This assay for acid-labile phosphate may therefore be generally useful to detect phosphoramidate linkages on proteins containing abundant phosphohydroxy amino acids. The amount of [^{32}P]ATP and inorganic [^{32}P]phosphate adhering to protein fixed in the $NaDodSO_4$–acrylamide gels and extracted during microdialysis is negligible as indicated by the control.

The ^{31}P NMR spectrum (Figure 2C) of an $NaDodSO_4$-quenched reaction mixture of H4 and crude nuclear isolates from Walker-256 reveals a pattern similar to the previous kinase reaction involving regenerating rat liver nuclei. However, the peak at 4.8 ppm corresponds well with standard τ-phosphohistidine and is absent in the control (Figure 2D).

Table I: ^{31}P NMR Chemical Shifts (ppm) of Phosphorylated Histidine Residues Relative to 85% Orthophosphoric Acid Reference at pH 9–10

species	ppm
τ-phosphohistidine	4.7[a]
π-phosphohistidine	4.8
π-phosphohistidine	5.4[a]
phosphorylated HPr, denatured	5.5[a]
	5.4[b]
phosphorylated H4, rat regenerating liver nuclei, denatured	5.4
phosphorylated H4, Walker-256, denatured	4.9

[a] Gassner et al. (1977). [b] Dooijewaard et al. (1979).

Levels of DNA remain undetected in both the reaction mixture and the control under the conditions used.

Phosphorylation of H4 by Phosphoramidate. The chemical phosphorylation of histidine residues is demonstrated by several methods. Acid lability is confirmed on $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography (Figure 4). Acid treatment of the chemically phosphorylated H4 rapidly hydrolyzes the phosphoramidate linkages (Figure 5). Standard τ-phosphohistidine coelutes with the major phosphorylated species present in base hydrolysates of chemically phosphorylated H4 on high-pressure liquid chromatography (Figure 6).

The ^{31}P NMR spectrum of $NaDodSO_4$-denatured chemically phosphorylated H4 reveals a prominent peak at 4.8 ppm which corresponds well with τ-phosphohistidine (Figure 7). A smaller peak at 6.1 ppm is also observed. Upon placement of phosphorylated H4 in low salt, two major resonances appear on the ^{31}P NMR spectrum (Figure 8). The single sharp peak at 4.8 ppm and with a line width of ~9 Hz still remains. The peak at 6.1 ppm has shifted to 7.3 ppm and has significantly increased in line width (to 55 Hz) and relative amplitude. When the chymotryptic fragment H4(38–102) is phosphorylated by phosphoramidate and subsequently analyzed by ^{31}P NMR under similar conditions, the major peak is broad and centered around 7.0 ppm. Phosphorylation of the H4(1–23) peptide by phosphoramidate and analysis by NMR under the same conditions yield a single sharp peak at 4.8 ppm and a peak corresponding to inorganic phosphate. The ^{31}P NMR

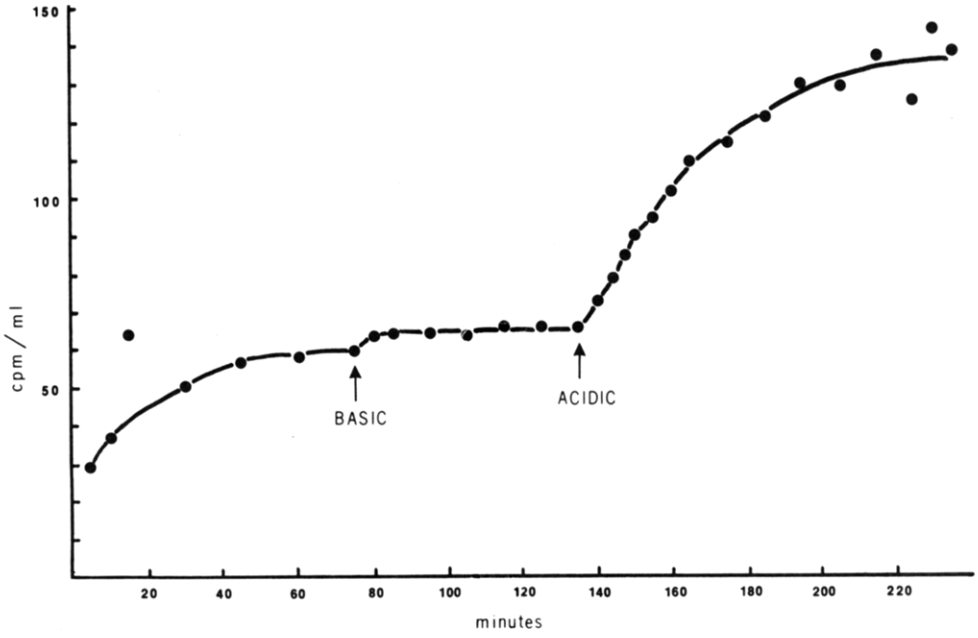

FIGURE 5: Determination of acid lability of chemically phosphorylated histone H4 using dialysis technique. The procedure was done as described under Materials and Methods, except 100 μg of whole histone was added to the enzymatic reaction instead of H4.

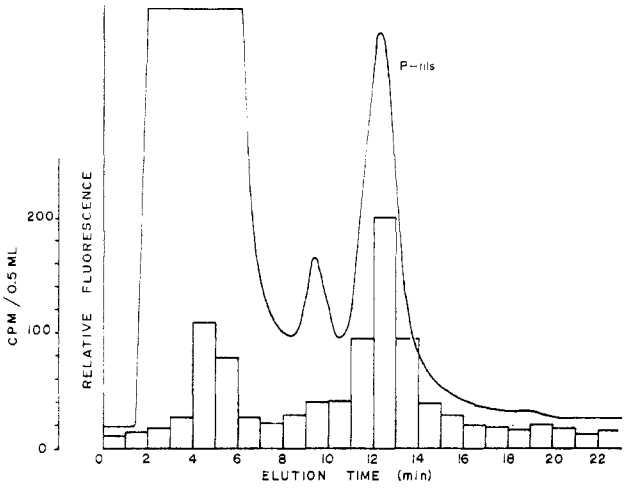

FIGURE 6: High-pressure liquid chromatography of base hydrolysates of chemically phosphorylated histone H4. HPLC sample preparation and analysis were done as described under Materials and Methods. The peak marked P-his coelutes with standard τ-phosphohistidine. Initial fluorescing peaks belong to other amino acids and free ammonia. The small radioactive peak eluting off at 5 min is residual [^{32}P]-phosphoramidate. Inorganic [^{32}P]phosphate elutes off after 40 min under these conditions.

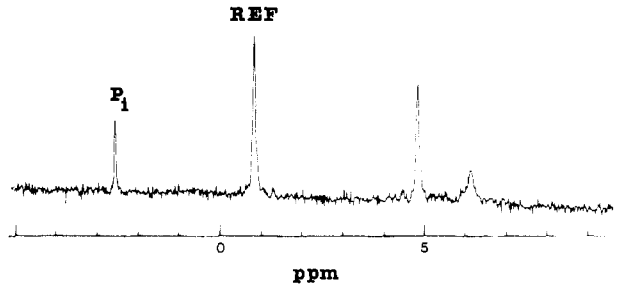

FIGURE 7: ^{31}P NMR of chemically phosphorylated histone H4 in 0.1% $NaDodSO_4$. NMR parameters are as described under Materials and Methods, except the number of transients is 13 000 and the pH is 9.3.

Table II: ^{31}P NMR Chemical Shifts (ppm) and Line Widths (Hz) of Chemically Phosphorylated H4 and Fragments at pH 7

species	ppm	Hz
phosphorylated H4, denatured	4.8 (6.1)	8
phosphorylated H4, low salt	4.8	9
	7.3	55
phosphorylated H4(1-23)	4.8	13
phosphorylated H4(38-102)	7.0	179

data are summarized in Tables I and II.

Discussion

Enzymatic Phosphorylation. There have been a number of ^{31}P NMR studies done on proteins with covalently bound phosphates. Most describe phosphorylation on the hydroxy moiety of serine [for example, see Otvos et al. (1979)]. The only phosphoramidate-containing protein studied thus far in any detail by NMR is the bacterial protein HPr involved in sugar transport (Schrecker et al., 1975). In theory, nuclear magnetic resonance of phosphate groups of completely denatured proteins should correspond well with those of phospoamino acid standards. There are, however, many factors which may invalidate such comparisons such as metal ion effects and steric strain. Gassner et al. (1977) and Dooijewaard et al. (1979) nevertheless both found good correlation of phosphohistidine model studies with denatured phosphorylated HPr, a protein known to contain phosphohistidine. At pH 11.8, HPr is denatured and the resonances found at 5.57

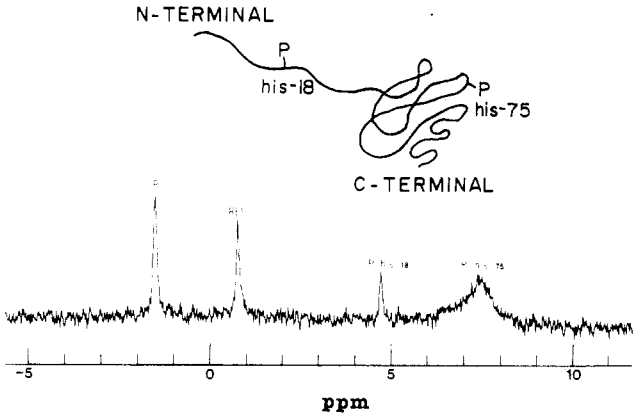

FIGURE 8: ^{31}P NMR of chemically phosphorylated histone H4. The above spectrum is representative of chemically phosphorylated H4 in <0.1 M NaCl. Higher salt concentrations caused aggregation, making NMR analysis difficult. The sample pH is 7 and the scanning temperature is 300 K. The number of transients is 17 000. Other NMR parameters are described under Materials and Methods.

and 5.4 ppm (relative to an external reference of 85% orthophosphoric acid) by Gassner et al. (1977) and Dooijewaard et al. (1979), respectively, were attributed to π-phosphohistidine which has a chemical shift of ~5.4 ppm at this pH.

^{31}P NMR is a useful technique to detect phosphoramidate bonds, since the number of manipulations of samples containing this labile linkage is kept to a minimum. Such linkages have been detected by other means in structural proteins such as myelin basic protein (Smith et al., 1976) and the histones (Smith et al., 1974). Phosphorylation occurring on basic amino acids of these basic proteins would undoubtedly affect their conformation and consequently their function. Phosphorylation of histidine has been shown to occur in vivo (Chen et al., 1974) and in vitro (Chen et al., 1977) on H4. The H4 kinase is present in cells that are rapidly turning over, and its peak of activity occurs during the onset of DNA transcription (Chen et al., 1977). Moreover, it has been demonstrated that the phosphorylation occurs on preformed H4 rather than newly synthesized H4 (Chen et al., 1977). Interestingly, H4 kinase derived from regenerating rat liver phosphorylates the π position of hisitidine whereas that derived from Walker-256 carcinosarcoma phosphorylates the τ position (Bruegger, 1977). The reason for these differences is not yet known. In addition, the phosphorylation of other amino acid residues on H4 was found to be negligible in regenerating rat liver nuclei (Garrard et al., 1976; Sung et al., 1971) and in Walker-256 nuclei (Bruegger, 1977).

The ^{31}P NMR data shown here are in agreement with these earlier findings. The spectrum of the regenerating rat liver H4 kinase reaction reveals a peak which corresponds well with standard π-phosphohistidine, and the spectrum of the Walker-256 carcinosarcoma H4 kinase reaction shows a peak which corresponds well with standard τ-phosphohistidine (Figure 2). The spectra of the controls (no added H4) show no phosphohistidine peak. The presence of a substantial amount of inorganic phosphate detected in the kinase reaction mixture may be attributed to chemical hydrolysis of the labile phosphoramidate linkage during NMR scanning. It is unlikely that peaks purported to be from phosphohistidine residues are resonances obtained from ATP binding to H4 since $NaDodSO_4$ is utilized and a control (data not shown) involving only H4 and ATP mixed under similar conditions shows no anomalous peaks. Resonances result solely from the reaction since no peaks are observed when H4 is scanned alone. The absence of a DNA resonance in the Walker-256 nuclear preparation may be attributed to the smaller quantity of extract added to

the reaction. While the isomer of phosphohistidine on proteins cannot be determined conclusively on ^{31}P NMR due to the close proximity of their resonances, the ^{31}P NMR studies conducted as well as the acid lability of the linkages are in complete agreement with previous findings.

Chemical Phosphorylation. The kinetic product of the reaction between the dipotassium salt of phosphoramidate and the imidazole residue of histidine is π-phosphohistidine whereas the thermodynamic product is τ-phosphohistidine (Hultquist et al., 1966). Phosphorylation using phosphoramidate has been done on several proteins thus far. Insulin (Rathlev & Rosenberg, 1956) and phosphoramidate–hexose transferase (Stevens-Clark et al., 1968) have been chemically phosphorylated, though the phosphorylated amino acid species was not well characterized in either case. Chemically phosphorylated HPr protein is believed to contain π-phosphohistidine on the basis of the inability of the phosphate intermediate to transfer phosphate (Gassner et al., 1977; Dooijewaard et al., 1979).

The histone H4 molecule consists of two major domains, a region containing mainly hydrophilic amino acid residues (N-terminal end) and a region containing mostly hydrophobic residues (C-terminal end) (DeLange et al., 1969). Each domain contains one histidine residue. Therefore, phosphorylation at these sites should serve as useful ^{31}P NMR probes to H4 structure.

By use of ^{32}P-labeled phosphoramidate to phosphorylate H4, chymotryptic cleavage (pH 8) of the product and isolation of the peptide fragments indicated that both histidines become phosphorylated (data not shown). Previous peptide analysis studies indicated that both histidine sites are in vitro enzymatically phosphorylated in H4 (Bruegger, 1977).

When chemically phosphorylated H4 is placed in a denaturing solvent, a single major peak at 4.8 ppm corresponding to τ-phosphohistidine is observed. Phosphorus groups at both histidine residues of the denatured H4 are probably contributing to this resonance. A smaller sharp peak at 6.1 ppm indicates that some secondary structure exists even in the presence of $NaDodSO_4$ and probably involves phosphorus at position 75 since this peak shifts and increases in size in low salt. When chemically phosphorylated H4 is placed in low salt, two major peaks appear, one sharp peak at 4.8 ppm and one broad peak at 7.3 ppm. As the phosphorylated H4 experiences folding and association, the line width and chemical shift remain fairly constant for the former peak whereas the amplitude, line width, and chemical shift increase significantly for the latter peak. Accurate quantitation is difficult due to the general lability of phosphoramidate bonds. High salt concentration causes aggregation, resulting in broadening of all resonances. These results may be interpreted as follows: both histidine residues of H4 are phosphorylated by phosphoramidate, and one of the phosphates encounters a more structured environment under low-salt conditions causing some immobilization and consequent line broadening. The other phosphate remains relatively mobile under similar conditions. This is in full agreement with the conformation studies of H4 (Crane-Robinson et al., 1977; Pekary et al., 1975): the carboxy-terminal end becomes structured in low salt whereas the amino-terminal end retains its unstructured state. H4 peptides representing the two major domains of the H4 molecule were prepared and subsequently phosphorylated with phosphoramidate to check this explanation. Fragments H4-(1–23) and H4(38–102), according to ^{1}H NMR studies (Lewis et al., 1975), exhibit similar behavior patterns in solvent to the two major domains of intact H4. Moreover, fragment H4(38–102) has a tendency to aggregate in salt solutions whereas fragment H4(1–23) does not. Indeed, in low salt a sharp resonance is observed for phosphorylated fragment H4(1–23) at 4.8 ppm indicating that the phosphoramidate group is relatively free in solution. The phosphorylated chymotryptic fragment H4(38–102) yields a very broad peak centered at 7.0 ppm. The chemical shift and line width of this peak indicate that the phosphoramidate group is in close proximity with other amino acid residues and is partially immobilized. The spectrum of the phosphorylated chymotryptic fragment is broader than that of the phosphohistidine at the histidine-75 resonance on intact H4. This is an indication of even more immobilization. The lack of repulsions between the positively charged N-terminal region may be the cause of this higher associative capacity of the chymotryptic fragments (Crane-Robinson et al., 1977).

The relaxation time employed was sufficiently large to allow one to correlate peak areas with relative concentrations of protein-bound phosphate. Differences in peak areas indicate that phosphorylation occurring on histidine-18 is more easily hydrolyzed than that occurring on histidine-75. The NMR spectrum of a sample of chemically phosphorylated H4, rerun after 1 week at 5 °C, showed a substantial decrease in the peak area of phosphorylation occurring at histidine-18 with a concurrent increase in the peak area of inorganic phosphate.

It should be noted that in the denatured state line widths of the phosphate probes are identical and differ when using the same spectrometer frequency only upon changing solvent conditions which induce conformational changes in the protein. Therefore, the major relaxation mechanism involved here is most likely dipole–dipole with protons of neighboring amino acid residues around histidine-75. The contribution, if any, of chemical shift anistropy to line width for this small phosphorylated protein (M_r = 11 300) was not determined since experiments at different frequencies [see Hull & Sykes (1975)] were not done.

In summary, ^{31}P NMR is a useful tool for probing the structure of acid-labile phosphorylated H4 and detects phosphohistidine in both chemically and enzymatically phosphorylated H4. However, the purpose of such phosphorylation in nature is unknown. Undoubtedly, phosphorylation of basic amino acids on the highly conserved histone H4 would affect its associative properties with other histones and DNA. Reconstitution experiments of phosphorylated H4 with other histones and DNA analyzed by ^{31}P And ^{1}H NMR as well as other techniques should yield informative data on the nature and function of phosphorylated H4.

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References

Arfmann, H.-A., & Bode, J. (1980) *Int. J. Biol. Macromol. 2*, 109.

Bohm, E. L., Strickland, W. N., Strickland, M., Thwaits, B. H., Van Der Westhuizen, D. R., & Von Holt, C. (1973) *FEBS Lett. 34*, 217.

Bruegger, B. B. (1977) Ph.D. Dissertation, University of California, Los Angeles, CA.

Burger, A. R., Lippard, S. J., Pantoliano, M. W., & Valentine, J. S. (1980) *Biochemistry 19*, 4139.

Chauveau, J., Moule, Y., & Rouiller, C. (1956) *Exp. Cell Res. 11*, 317.

Chen, C.-C., Smith, D. L., Bruegger, G. G., Halpern, R. M., & Smith, R. A. (1974) *Biochemistry 13*, 3785.

Chen, C.-C., Bruegger, B. B., Kern, C. W., Lin, Y. C., Halpern, R. M., & Smith, R. A. (1977) *Biochemistry 16*, 4852.
Crane-Robinson, C., Hayashi, H., Cary, P. D., Briand, G., Sautiere, P., Krieger, D., Vidali, G., Lewis, P. N., & Tom-Kun, J. (1977) *Eur. J. Biochem. 79*, 535.
DeLange, R. J., Fambrough, D. M., Smith, E. L., & Bonner, J. (1969) *J. Biol. Chem. 244*, 319.
Dooijewaard, G., Roossien, F. F., & Robillard, G. T. (1979) *Biochemistry 18*, 2996.
Dower, S. K., & Dwek, R. A. (1979) *Biochemistry 18*, 3668.
Fischer, S. G., & Laemmli, U. K. (1980) *Biochemistry 19*, 2240.
Garrard, W. T., Kidd, G. H., & Bonner, J. (1976) *Biochem. Biophys. Res. Commun. 70*, 1219.
Gassner, M., Stehlik, D., Schrecker, O., Hengstenberg, W., Maureer, W., & Ruterjans, J. (1977) *Eur. J. Biochem. 75*, 287.
Goetze, A. M., & Richards, J. H. (1977) *Biochemistry 16*, 228.
Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., & Tobey, R. A. (1978) *Eur. J. Biochem. 84*, 1.
Higgins, G. M., & Anderson, R. M. (1931) *Arch. Pathol. 12*, 186.
Hull, W. E., & Sykes, B. D. (1975) *J. Mol. Biol. 98*, 121.
Hultquist, D. E., Moyer, R. W., & Boyer, P. D. (1966) *Biochemistry 5*, 322.
Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Langan, T. A. (1978) *Methods Cell Biol. 14*, 127.
Lewis, P. N., Bradbury, E. M., & Crane-Robinson, D. (1975) *Biochemistry 14*, 3391.
Lin, Y.-C., Rose, K. M., & Jacob, S. T. (1976) *Biochem. Biophys. Res. Commun. 72*, 115.
O'Brien, R. D. (1960) *Toxic Phosphorus Esters*, p 347, Academic Press, New York.
Otvos, J. D., Armitage, I. M., Chlebowski, J. F., & Coleman, J. E. (1979) *J. Biol. Chem. 254*, 4707.
Pekary, A. E., Li, H.-J., Chan, S. I., Hsu, C.-J., & Wagner, T. E. (1975) *Biochemistry 14*, 1177.
Rao, B. D. N., Cohn, M., & Noda, L. (1978) *J. Biol. Chem. 253*, 1149.
Rathlev, T., & Rosenberg, T. (1956) *Arch. Biochem. Biophys. 65*, 319.
Schlitz, E., & Sekeris, C. E. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 317.
Schnackerz, K. D., Feldmann, K., & Hull, W. E. (1979) *Biochemistry 18*, 1536.
Schrecker, O., Stein, R., Hengstenberg, W. Gassner, M., & Stehlik, D. (1975) *FEBS Lett. 51*, 309.
Smith, D. L., Bruegger, B. B., Halpern, R. M., & Smith, R. A. (1973) *Nature (London) 246*, 103.
Smith, D. L., Chen, C.-C., Bruegger, B. B., Holtz, S. L., Halpern, R. M., & Smith, R. A. (1974) *Biochemistry 13*, 3780.
Smith, L. S., Kern, C. W., Halpern, R. M., & Smith, R. A. (1976) *Biochem. Biophys. Res. Commun. 71*, 459.
Smith, R. A., & Theisen, M. C. (1967) *Methods Enzymol. 4*, 403.
Smith, R. A., Halpern, R. M., Bruegger, B. B., Dunlap, A. K., & Fricke, O. (1978) *Methods Cell Biol. 14*, 153.
Steiner, A. W., Helander, E. R., Fujitaki, J. M., Smith, L. S., & Smith, R. A. (1980) *J. Chromatogr. 202*, 263.
Stevens-Clark, J. R., Conklin, K. A., Fujimoto, A., & Smith, R. A. (1968) *J. Biol. Chem. 243*, 4473.
Stokes, H. N. (1893) *Am. Chem. J. 15*, 198.
Sung, M. T., Dixon, G. H., & Smithies, O. (1971) *J. Biol. Chem. 246*, 1358.
Sykes, B. D., & Hull, W. E. (1978) *Methods Enzymol. 49*, 270.
Szopa, J., Jacob, G., & Arfmann, H.-A. (1980) *Biochemistry 19*, 987.
Weintraub, H., Palter, K., & Van Lente, F. (1975) *Cell (Cambridge, Mass.) 6*, 85.
Zetterqvist, O., & Engstrom, L. (1966) *Biochim. Biophys. Acta 113*, 520.