Intramolecular Localization and Effect on Conformational Stability in Vitro of Irreversible Interphase Phosphorylation of *Physarum* Histone H1†

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ABSTRACT: To elucidate the intramolecular localization of irreversible interphase phosphorylation of *Physarum* histone H1 [Jerzmanowski, A., & Maleszewski, M. (1985) *Biochemistry 24*, 2360–2367] and its effect on H1's conformational properties, the circular dichroism spectra, the pH- and salt-dependent folding, and the products of trypsin digestion for the interphase phosphorylated (with five to nine phosphates per molecule) and enzymatically dephosphorylated H1 were compared. Both phosphorylated and dephosphorylated H1 show similar amounts (6.2 and 5.5%, respectively) of helicity at high ionic strength and upon limited digestion with trypsin form identical trypsin-resistant peptides of the size slightly larger than the analogous peptide from calf thymus H1. The circular dichroism analysis of the pH-dependent folding of *Physarum* H1 in water shows a strong effect of phosphorylation on the folding process in both the acidic and alkaline pH region. The analysis of the products of trypsin digestion of [^{32}P]PO_4-labeled *Physarum* H1 before and after enzymatic dephosphorylation is consistent with the interpretation that the interphase phosphorylation occurs predominantly within the 50–70 amino acid sequence directly adjacent to the trypsin-resistant peptide on its C-terminal side and that this sequence is itself involved in some kind of loose folding at high ionic strength. The studies of the formation of the trypsin-resistant peptide (the globular domain) as a function of salt concentration show that it is induced at 300 mM lower NaCl concentration for phosphorylated than for dephosphorylated H1. These results indicate that the stable, interphase phosphorylation of *Physarum* H1 enhances the salt-induced formation of the folded globular region in vitro. This conclusion together with our finding that only nonphosphorylated H1 occurs in the DNase I solubilized fraction of *Physarum* chromatin may be relevant for a mechanism of chromatin activation in *Physarum*.

It is generally accepted that H1 histones are involved in the formation of higher order chromatin structure (Thoma et al., 1979). Most organisms contain more than one H1 protein (termed H1 subtypes), and the relative proportions of subtypes present depend on the cell type as well as the stage of development (Cole, 1984). Additional variation is introduced by the postsynthetic modifications of H1 proteins, mostly by phosphorylation at Ser and Thr (Hohmann, 1983). Since the degree of chromatin condensation is critical for its transcriptional activity, it has been suggested that the different H1 subtypes (which can be phosphorylated to a different extent and at different sites), by affecting the state of chromatin condensation differently, may function as coarse regulators of genetic expression (Cole, 1984).

In histone H1 only the central hydrophobic region folds into globular structure upon an increase in salt concentration while the highly basic N- and C-terminal flanks remain extended (Bradbury et al., 1965; Chapman et al., 1976; Aviles et al., 1978). The globular region of H1 is well conserved among species and identical for H1s of the same organism. The differences in amino acid sequence are on the other hand abundant in the polycationic flanks (Cole, 1984). It has been suggested that the globular domain of H1, by forming a bridge between the two coils of DNA wrapped around the nucleosome, is essential for maintaining the path of internucleosomal DNA, thus allowing the regular higher order chromatin organization under proper ionic conditions (Allan et al., 1980; Chan et al., 1984). On the other hand it has been shown for H1 in solution that the folding of the hydrophobic region is influenced by primary sequence differences in extended flanks (Smerdon & Isenberg, 1976) and also that the subtypes of H1 differ in their ability to condense DNA when studied by filter binding, sedimentation, and viscosity (Welch & Cole, 1979, 1980; Liao & Cole, 1981). However, more evidence is still necessary to prove the role of the variation in H1 subtypes in the regulation of genetic expression.

We have recently shown (Jerzmanowski & Maleszewski, 1985) that in the lower eucaryote *Physarum polycephalum* histone H1 is represented by a single sequence variant that undergoes an unusual type of postsynthetic modification during the mitotic cycle. In addition to the irreversible methylation of the ϵ-NH_2 group of several of its lysines, *Physarum* H1 becomes irreversibly phosphorylated by accepting 8–12 phosphate groups per molecule. This irreversible phosphorylation concerns 100% of the newly synthesized H1 and is considerably delayed with respect to the deposition of H1 on the chromatin DNA. It begins at the end of the S phase and lasts until about one-third of the G2 phase (*Physarum* lacks the G1 phase). We have also confirmed the occurrence in *Physarum* of the reversible, cyclic superphosphorylation of H1 during mitosis, which is superimposed on the irreversible interphase event.

In the present work we studied the intramolecular localization of the irreversible interphase phosphorylation and its effect on the conformational properties of *Physarum* H1 in solution. We have found that the interphase phosphorylation does not occur in the globular domain but has a strong effect on its salt-induced folding in vitro.

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Materials and Methods

Physarum Culture and Labeling. The conditions of culturing of *Physarum microplasmodia* and labeling with [^{32}P]PO_4 were as described before (Jerzmanowski & Maleszewski, 1985).

Preparation of Histones. Histone H1 was isolated from microplasmodial nuclei or chromatin by the guanidine hydrochloride procedure described by Mende et al. (1983) with additional purification of the final preparation by the extraction with 5% perchloric acid. Lyophilized H1 was then dissolved in 0.02 M HCl and further purified by chromatography on a 1.5 × 160 cm Sephadex G-100 column eluted with the same solution. Protein in the effluent was detected by absorbance at 275 nm. The first two-thirds of H1 peak was collected and lyophilized.

The crude preparation of calf thymus H1 was isolated by the NaCl–citrate method of Johns (1964) and further purified by chromatography on a 2 × 80 cm Bio-Gel P-100 column eluted with 0.02 M HCl. The final preparation was composed of two main H1 subfractions and was free of contaminations with other proteins as judged by polyacrylamide gel electrophoresis.

Dephosphorylation of Physarum H1 with Alkaline Phosphatase. Column-purified *Physarum* H1 was digested with alkaline phosphatase from *Escherichia coli* (P-L Biochemicals) as described before (Jerzmanowski & Maleszewski, 1985). After 1.5 h the reaction was stopped by the addition of cold perchloric acid to the final concentration of 5%. After 1 h on ice the precipitate of alkaline phosphatase was centrifuged. The H1-containing supernatant was dialyzed extensively to 0.02 M HCl and applied on a 0.9 × 40 cm Bio-Gel P-100 column equilibrated with 0.02 M HCl. The column was eluted with the same solution and the protein in the effluent was detected by absorbance at 275 nm. The H1 peak was collected and lyophilized. In the experiments with trypsin digestion of [^{32}P]PO_4-labeled H1 dephosphorylated with alkaline phosphatase the Bio-Gel column was omitted as it was found that the remaining traces of alkaline phosphatase do not interfere with trypsin digestion of H1.

Digestion with Proteolytic Enzymes and Isolation of Histone Fragments. For the digestion with trypsin *Physarum* histone H1 was dissolved in 2 M NaCl and 50 mM Tris-HCl (pH 8.0), and a TPCK-treated trypsin (a gift from Serva, Heidelberg) was then added to obtain an enzyme:histone ratio of 1:1000 (w/w). Digestion was carried out at 21 °C and was terminated by the addition of 100% (w/v) trichloroacetic acid to the final concentration of 25%. After 2–3 h on ice the precipitate was centrifuged, washed with cold acetone, and dissolved in a minimal amount of water for analysis by NaDodSO$_4$–polyacrylamide gel electrophoresis.

For the isolation of the intermediate products of trypsin digestion (P1), the reaction with trypsin was stopped after 5 min by lowering the pH to 2.0 with diluted HCl and adding of soybean trypsin inhibitor to give an inhibitor:trypsin ratio of 2:1 (w/w). The reaction mixture was then applied on 0.9 × 40 cm Bio-Gel P-30 column and eluted with 0.02 M HCl and 50 mM NaCl. One-tenth of each fraction was used for analysis by NaDodSO$_4$–polyacrylamide gel electrophoresis. The pooled fractions containing the P1 products of digestion were adjusted to pH 8.0 and 2 M NaCl and redigested with trypsin as described above. Digestion of *Physarum* H1 with chymotrypsin was as described before (Jerzmanowski & Maleszewski, 1985).

The trypsin-resistant peptide of calf thymus H1 was prepared as described by Barbero et al. (1980) and purified by chromatography on Sephadex G-50 as described by Hartman et al. (1977).

Fractionation of Physarum Chromatin with DNase I. Nuclei from log-phase microplasmodia were suspended in 0.25 M sucrose, 0.1 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.8) and digested with 1×10^{-3} μg/mL DNase I (Worthington) to give 1–3% acid-soluble DNA. After the mixture was chilled and EDTA was added to 5 mM, the mixture was centrifuged and histone H1 was isolated from the solubilized and pelleted nucleoprotein by the guanidine hydrochloride procedure described above.

Circular Dichroism Measurements. Circular dichroism spectra were measured at 22 °C in a 10-mm pathlength cell with a Mark III dicrograph (Jobin-Yvone) operated at a sensitivity of 2×10^{-6} ΔA mm^{-1}. Stock solutions of calf thymus H1 for circular dichroism measurements were prepared by dissolving H1 at 1.0 mg/mL in water. The H1 concentration in stock was determined from the absorption at 275 nm using an absorbance coefficient of 1345 cm^{-1} M^{-1} (Smerdon & Isenberg, 1976). Absorbance spectra were measured with a Cary 118 spectrophotometer. Stock solutions of *Physarum* H1 were prepared by dissolving the phosphorylated and dephosphorylated H1 at 0.5 mg/mL in water. The concentration in stocks was determined by the procedure of Lowry et al. (1951) using calf thymus H1 as standard. The solutions of H1 for circular dichroism measurements were prepared by mixing in the measuring cell the proper volumes of stock H1, water, either 0.1 M HCl (to prepare the pH 2 solution) or 4 M KF, and 25 mM Tris-HCl (pH 8.0) (to prepare the high ionic strength solution). For measurements at different pH, samples were brought to pH 2.8 with diluted HCl before adjustment to the final volume and the pH was raised by adding predetermined aliquots of diluted NaOH.

Analytical Techniques. NaDodSO$_4$–polyacrylamide gradient gel electrophoresis and autoradiography were performed as described before (Jerzmanowski & Maleszewski, 1985). Densitometer scans of Coomassie blue stained gels were done with a Zeiss–Jena densitometer.

Results

Preparation of Enzymatically Dephosphorylated H1 and Comparison with H1 from Chromatin Fractionated with DNase I. Physarum histone H1 to be used for circular dichroism studies was isolated by the guanidine hydrochloride method from log-phase microplasmodia and purified by chromatography on a Sephadex G-100 column to more than 95% purity as judged by electrophoresis in NaDodSO$_4$–polyacrylamide gels (Figure 1, upper panel, lane B). Column-purified H1 was dephosphorylated in vitro with alkaline phosphatase from *E. coli* and again purified by selective extraction with 5% perchloric acid and column chromatography on a Bio-Gel P-100 column (Figure 1, upper panel, lane A). The effect of in vitro dephosphorylation was a marked decrease of the heterogenity of the H1 band in gel electrophoresis. That this reflected an almost complete removal of phosphates from H1 could be judged from similar experiment with H1 isolated from cultures grown with radioactive phosphate (Figures 4 and 5, lane A).

In another type of experiment we analyzed the distribution of H1 in *Physarum* chromatin fractionated with nucleases. Figure 1 (lower panel, lane B′) shows the pattern of H1 isolated from the fraction of chromatin released from nuclei by limited digestion with DNase I. Although the general properties of this fraction corresponded to those of "active chromatin" (Czupryn & Toczko, 1985), its selective enrichment in freshly replicated chromatin could not be excluded.

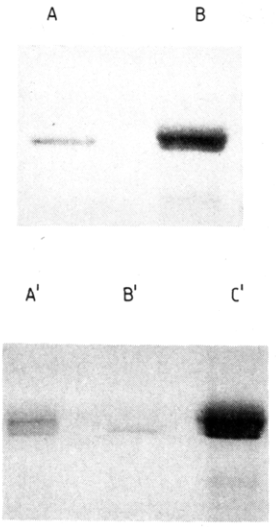

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of *Physarum* histone H1. (Upper panel) Samples used for circular dichroism studies: (A) H1 dephosphorylated with alkaline phosphatase; (B) H1 from log-phase microplasmodia. (Lower panel) H1 isolated from *Physarum* chromatin fractionated by limited digestion with DNase I: (A′) H1 from nuclear pellet after DNase I digestion; (B′) H1 from nucleoprotein released to supernatant after DNase I digestion; (C′) H1 from unfractionated nuclei.

The fraction amounted to 6–10% of the total chromatin. It is seen from Figure 1 that the appearance in $NaDodSO_4$ gel of H1 from the DNase I released fraction is indistinguishable from that of enzymatically dephosphorylated H1. The H1 from remaining nuclear chromatin as well as from unfractionated nuclei (Figure 1, lower panel, lanes A′ and C′) has a typical heterogeneous appearance (slightly more expanded than in lane B, upper of Figure 1, due to a higher resolution of electrophoresis).

Circular Dichroism of Phosphorylated and Dephosphorylated Physarum H1. The main difference between calf thymus and *Physarum* H1 revealed by circular dichroism spectra was a significantly lower percent of secondary structure characteristic for the *Physarum* protein (Figure 2). The maximum value of the $[\Theta]_{222}$ extremum at high ionic strength was −4000 for calf thymus H1 and only −2800 and −2600 for phosphorylated and dephosphorylated *Physarum* H1, respectively. On the basis of a random coil ellipticity of −1000 as an average for histones and 8 M urea (Crane-Robinson et al., 1976) and a fully helical ellipticity of −30 000 (Chen et al., 1974), this corresponds to 10.3% helicity for calf thymus H1 and 6.2% and 5.5% helicity for phosphorylated and dephosphorylated *Physarum* H1, respectively. These values are in good agreement with the α-helix content reported for the total *Physarum* H1 by Cary et al. (1985) on the basis of their circular dichroism studies. The lower value of $[\Theta]_{222}$ in water at pH 2 for phosphorylated than for dephosphorylated *Physarum* H1 (Figure 2) indicates that the phosphorylated form of the protein is more resistant to acidic denaturation.

Effect of pH on the Secondary Structure of Phosphorylated and Dephosphorylated Physarum H1. Figure 3 shows the pH dependence of the stability of the secondary structure in water for phosphorylated and dephosphorylated *Physarum* H1 studied by circular dichroism measurement at 222 nm. The results for calf thymus H1 are shown for comparison. Both the calf thymus and the dephosphorylated *Physarum* H1 show similar variation in their secondary structure between pH 2 and 12. A sharp conformational transition occurs in both histones between pH 7 and 10 with a midpoint at about pH 8. There is no change in the amount of adopted secondary

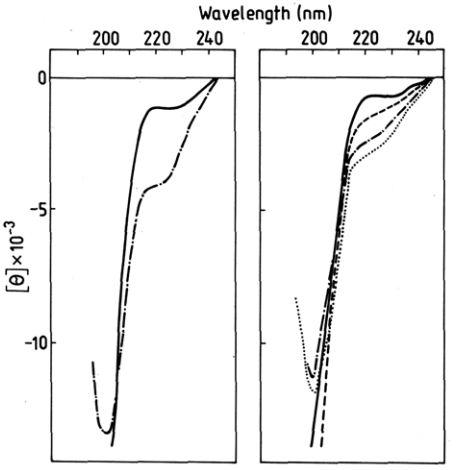

FIGURE 2: Circular dichroism spectra of calf thymus and *Physarum* histone H1. Left panel: spectra for calf thymus H1 (—) in water, pH 2.0; (–·–) in 2 M KF and 25 mM Tris-HCl (pH 8.0). Right panel: spectra for Physarum H1 (—) dephosphorylated, in water, pH 2.0; (–·–) dephosphorylated, in 2 M KF and 25 mM Tris-HCl (pH 8.0); (– –) phosphorylated, in water, pH 2.0; (···) phosphorylated, in 2 M KF and 25 mM Tris-HCl (pH 8.0). Spectra were recorded at 22 °C in a 10-mm path-length cell at a concentration of 8.7 μg/mL for calf thymus H1 and 6.4 and 6.7 μg/mL for phosphorylated and dephosphorylated *Physarum* H1, respectively. Results are expressed as molar ellipticity with the dimension of deg cm^2 (dmol of residue)$^{-1}$.

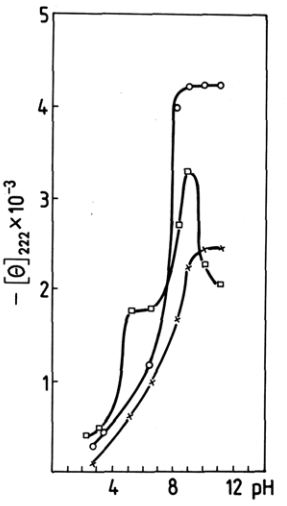

FIGURE 3: Variation of $[\Theta]_{222}$ with pH for water solutions of (O) calf thymus H1, (×) dephosphorylated *Physarum* and H1, (□) phosphorylated *Physarum* H1. Conditions of measurement were as described in Figure 2.

structure in the alkaline pH region up to pH 12. These results are in good agreement with earlier data for H1s from different sources (Barbero et al., 1980). However, a significantly different pattern is observed for *Physarum* H1 in the phosphorylated form. The conformational transition occurs earlier on a pH scale and is biphasic with a first midpoint at pH 4.5 and the second at pH 8. The adopted secondary structure is no longer stable in the alkaline pH region and a sharp denaturation is observed starting from pH between 9 and 10.

From the results of Figure 3 it can be concluded that the presence of phosphates exerts a strong effect on the structuralization of *Physarum* H1. This could mean that the phosphate groups were either within the structured domain or in a position that facilitated their interaction with the folding region.

Analysis of Physarum H1 by Limited Digestion with Trypsin. We have previously established (A. Jerzmanowski and A. M. Krężel, unpublished results) that the limited trypsin

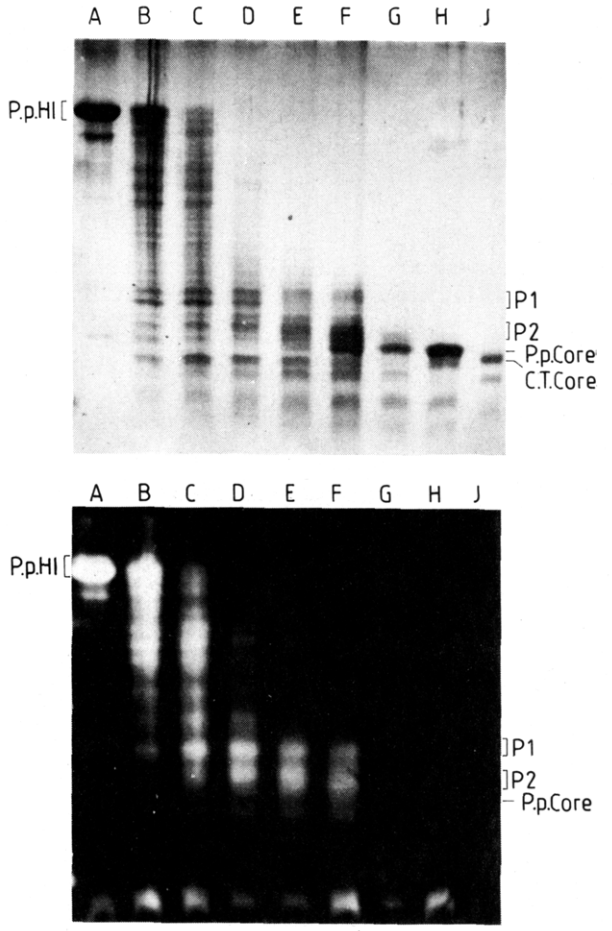

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of the products of limited digestion with trypsin of [^{32}P]PO_4-labeled *Physarum* histone H1 from log-phase microplasmodia. H1 from cultures grown in the presence of [^{32}P]PO_4 was digested with TPCK–trypsin at the ratio of enzyme to protein of 1:1000. Samples were taken in the course of digestion and analyzed by gel electrophoresis. (Upper panel): Coomassie blue stained gel. (Lower panel): corresponding autoradiogram: (A) undigested sample; (B) 3 min; (C) 5 min; (D) 10 min; (E) 20 min; (F) 30 min; (G) 60 min; (H) 120 min of digestion; (J) trypsin-resistant peptide from calf thymus H1. P.p. H1, position of *Physarum* H1. P1 and P2, positions of intermediate products of digestion. P.p. core, position of trypsin-resistant peptide from *Physarum* H1. C.T. core, a position of trypsin-resistant peptide from calf thymus H1.

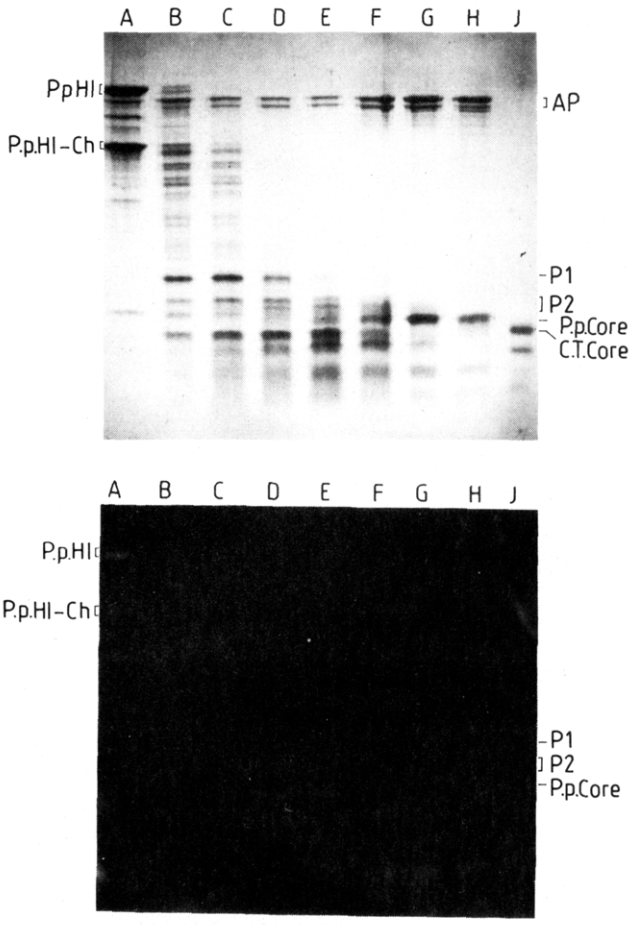

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of the products of limited digestion with trypsin of [^{32}P]PO_4-labeled *Physarum* histone H1 from log-phase microplasmodia, dephosphorylated in vitro with alkaline phosphatase. Conditions of dephosphorylation and digestion with trypsin as described under Materials and Methods and in legend to Figure 4. (Upper panel): Coomassie blue stained gel. (Lower panel): corresponding autoradiogram: (A) undigested sample; (B) 3 min; (C) 5 min; (D) 10 min; (E) 20 min; (F) 30 min; (G) 60 min; (H) 120 min of digestion; (J) trypsin-resistant peptide from calf thymus H1. P.p. H1-Ch, position of chymotryptic fragment of *Physarum* histone H1 generated in the course of dephosphorylation with alkaline phosphatase. AP, position of alkaline phosphatase. Other descriptions as in legend to Figure 4.

digestion at high ionic strength of *Physarum* histone H1 in both the phosphorylated and dephosphorylated form results in a trypsin-resistant peptide identical for both forms and slightly larger than the analogous peptide from calf thymus H1. In order to examine the distribution of modificating phosphate groups with respect to the trypsin-resistant peptide, we isolated histone H1 from log-phase cultures grown for 24 h in the presence of [^{32}P]PO_4 and digested it with trypsin before and after dephosphorylation with alkaline phosphatase. The progress of digestion as monitored by $NaDodSO_4$ gel electrophoresis of the reaction products is shown in the upper panels of Figure 4 (for phosphorylated) and Figure 5 (for dephosphorylated) H1. The lower panels of Figures 4 and 5 show the corresponding autoradiograms. The small amount of lower molecular weight protein seen directly below the main H1 band in lanes A results from a nonenzymatic cleavage of a short portion of the C-terminal region of *Physarum* H1 (Cote & Pallota, 1985). It is seen from Figure 4 that the interphase phosphorylation of *Physarum* H1 does not occur in the trypsin-resistant peptide. This is evident from comparison of electrophoretic patterns of lanes G and H, where the trypsin-resistant peptide has already accumulated, with the corresponding autoradiogram.

Although it is difficult to analyze quantitatively the results of this type of experiment, it seems safe to conclude that the radioactivity from H1 accumulates predominantly in the two intermediate, heterogeneous products designated as P1 and P2 on Figures 4 and 5. The radioactive band seen on the bottom of the autoradiogram of Figure 4 does not correspond to any Coomassie blue stained band on the original gel. It could be the inorganic phosphate resulting from unspecific hydrolysis of some phosphates from H1 during boiling of the samples prior to electrophoresis.

To clarify the relation between the P1 and P2 groups of products, we isolated by chromatography on a Bio-Gel P-30 column the P1 group of peptides from a partly digested sample of H1. The redigestion of isolated P1 products with trypsin gave P2 products that were subsequently transformed into the trypsin-resistant peptide of a size identical with that formed from the intact H1 (Figure 6). We therefore concluded that both the P1 and the P2 intermediate products contained the trypsin-resistant peptide. As more than 95% of the radioactivity present in H1 used for the trypsin digestion experiments

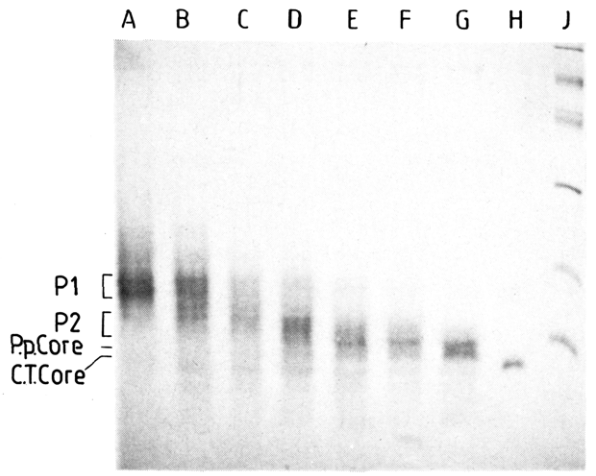

FIGURE 6: Redigestion with trypsin of the P1 product isolated from partially digested *Physarum* H1 and purified by chromatography on Bio-gel P-30: (A) undigested P1; (B) 5 min; (C) 10 min; (D) 20 min; (E) 30 min; (F) 40 min; (G) 60 min of digestion; (H) trypsin-resistant peptide from calf thymus H1; (J) molecular weight standards. Conditions of digestion and other descriptions as in legend to Figure 4.

remained in the large C-terminal chymotryptic fragment of the molecule upon digestion with chymotrypsin [results not shown; see also Jerzmanowski and Maleszewski (1985)], we further concluded that the phosphate-rich P1 and P2 products are composed of the trypsin-resistant peptide and the adjacent sequences of H1 extending toward the C-terminus. Both the precursor–product relationship and the analysis of the amount of radioactivity present in P1 and P2 when both occur at roughly the same concentration (lane D on the electrophorogram and autoradiogram of Figure 4) suggest that upon the transition from P1 to P2 the majority of the phosphates remains in P2. By comparison of the position of P1 in Na-DodSO$_4$–polyacrylamide gel electrophoresis with the positions of the molecular weight markers and histones, we concluded that its molecular weight is in the range 16 000–18 000, which corresponds to 180–200 amino acids. This evaluation is based on the assumption that the behavior of P1 in denaturing gels resembles that of core histones rather than intact H1. Indeed in both P1 and core histones the unstructured flanks are much shorter than those of H1 and in addition the flanking sequence in P1 has some tendency to fold (see below). Nevertheless the evaluation of the exact size will have to wait for the sequencing of the whole *Physarum* H1.

As can be seen from Figures 4 and 5, both the P1 and P2 peptides show some resistance to trypsin independently of whether phosphorylated. To see whether this resulted from the less frequent occurrence of trypsin cutting sites or from structural reasons we analyzed the occurrence of P1 and P2 upon digestion with trypsin as a function of NaCl concentration. The digestion time was selected that gave the accumulation of P1 and P2 under high ionic strength conditions. The results (Figure 7) show that the occurrence of both groups of peptides is dependent on the presence of salt. This indicates some degree of structuralization in the region of *Physarum* H1 directly adjacent to the trypsin-resistant peptide on its C-terminal side. Assuming the length of the N-terminal fragment and the trypsin-resistant peptide to be 130 amino acids in sum (Cary et al., 1985), we estimate the length of the C-terminal region contained in the P1 product to be 50–70 amino acids.

Effect of Interphase Phosphorylation on Folding of Physarum H1. To elucidate the influence of interphase phosphorylation on the ability of H1 to adopt a stable, folded

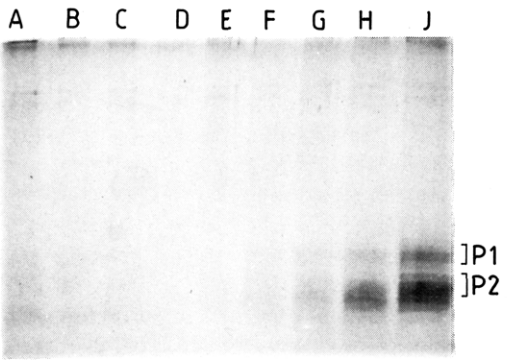

FIGURE 7: Digestion of *Physarum* histone H1 from log-phase microplasmodia with trypsin at different salt concentrations. *Physarum* H1 was incubated for 20 min with TPCK–trypsin at the enzyme to protein ratio of 1:1000 at pH 8.0 in the presence of different NaCl concentrations. Samples were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. Concentrations of NaCl in the digestion mixture: (A) 0.050 M; (B) 0.160 M; (C) 0.230 M; (D) 0.350 M; (E) 0.500 M; (F) 0.580 M; (G) 0.780 M; (H) 1.150 M; (I) 2.000 M. P1 and P2, positions of intermediate products of digestion.

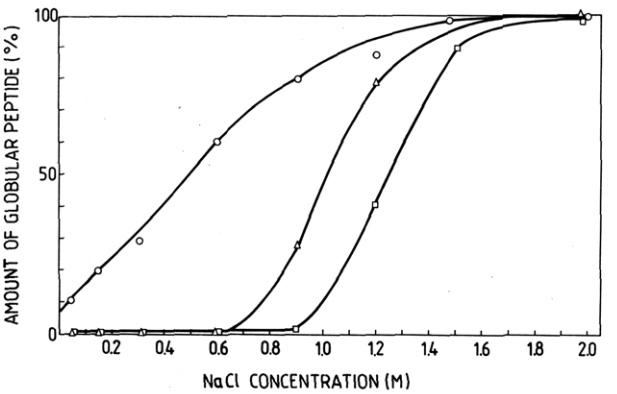

FIGURE 8: Accumulation of trypsin-resistant peptide after 60 min of digestion of histone H1 with trypsin as a function of NaCl concentration: (○) calf thymus H1; (△) phosphorylated *Physarum* H1; (□) dephosphorylated *Physarum* H1. Histones were digested with TPCK–trypsin at an enzyme to protein ratio of 1:1000 for 60 min and the products analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. The amount of trypsin-resistant peptide was estimated from the area of the corresponding peak on densitometer scans of Coomassie blue stained gels and expressed as percent peak area for 2.0 M NaCl.

conformation we estimated the amount of the trypsin-resistant peptide formed from phosphorylated and dephosphorylated H1 upon 60-min digestion at the same enzyme:protein ratio as a function of salt concentration. The estimates were based on measurements of the area of the peak corresponding to the trypsin-resistant peptide on densitometer scans of NaDodSO$_4$ gels. The results shown in Figure 8 are expressed as percent trypsin-resistant peptide formed at 2 M NaCl. Data for calf thymus H1 are shown for comparison. It is seen that for the phosphorylated *Physarum* H1 the formation of stable, trypsin-resistant peptide begins at about 300 mM lower NaCl concentration (between 0.6 and 0.9 M) than for the dephosphorylated H1 (between 0.9 and 1.2M). This difference indicates that there is a pronounced enhancing effect of the modificating phosphate groups on the salt-induced folding of H1. The comparison with the curve for calf thymus H1 shows that the stability of the folded domain in *Physarum* H1 is in general considerably more dependent on the presence of salt.

Discussion

Physarum histone H1 is unique in several respects: (1) With about 320 amino acids (Cary et al., 1985) it is on average 30%

larger than the H1 histones known so far. (2) Unlike all other H1 histones studied so far, it carries the irreversible, post-synthetic modifications by methylation and phosphorylation that occur independently on the reversible, cyclic super-phosphorylation in mitosis (Jerzmanowski & Maleszewski, 1985). (3) It occurs in *Physarum* chromatin as a single-sequence variant (Jerzmanowski & Maleszewski, 1985).

In this work we compared the properties of purified *Physarum* H1 carrying the stable, interphase phosphorylation with those of enzymatically dephosphorylated H1. The phosphorylated H1 was isolated from the log-phase cultures with the majority of nuclei in G2 phase. The predominant fraction of H1 in this preparation carries five to nine phosphates per molecule (Jerzmanowski & Maleszewski, 1985).

The circular dichroism studies of phosphorylated and dephosphorylated *Physarum* H1 revealed that in both forms a similar percent of amino acids is involved in the formation of the secondary structure (Figure 2). The determined amount of helicity (6.2% for phosphorylated and 5.5% for dephosphorylated as compared to 10.3% for calf thymus H1) corresponds well to the value established recently by Cary et al. (1985) on the basis of circular dichroism studies on the total, microplasmodial H1 from *Physarum*. On the other hand both the phosphorylated and dephosphorylated *Physarum* H1 upon limited digestion with trypsin at high ionic strength (2 M NaCl) produced a stable, trypsin-resistant peptide of a size even slightly larger than that of an analogous peptide from calf thymus H1 (Figures 4 and 5). Therefore the smaller percent of helicity revealed by circular dichroism is probably largely due to the increased size of *Physarum* H1 molecule. It can be thus concluded that in both the phosphorylated and dephosphorylated form of *Physarum* H1 a stable globular domain exists at high ionic strength and its size is similar to that of the globular domains in typical mammalian H1s. An important difference between the phosphorylated and dephosphorylated form was however noticed in studies of the pH-dependent folding in water (Figure 3). The measurements of $[\Theta]_{222}$ vs. pH showed a strong effect of the modificating phosphates on the folding process. Whereas for dephosphorylated *Physarum* H1 the folding curve resembled closely that for calf thymus H1 with a midpoint of a single cooperative transition at about pH 8 and a conformational stability fully maintained in the alkaline pH region, the phosphorylated H1 showed a biphasic conformational transition with the first midpoint at pH 4.5, and it denatured sharply in the alkaline pH region. The above differences can be explained by the presence of additional negative groups in phosphorylated H1. It has been noted that the core peptide of H1 produced by cutting off the highly basic N- and C-terminal flanks (i.e., with greatly decreased overall positive charge) folds at about 1 unit lower pH than the intact molecule (Barbero et al., 1980). A much more pronounced effect (about 3.5 units lower pH of folding) of only five to nine phosphate groups introduced within highly basic surroundings and only partly ionized at acidic pH suggests a specific position of the negative charges with respect to the folding domain. The picture is similar for the denaturation in the alkaline pH region. At this pH the titration of lysine residues is almost complete and the hydrophobically stabilized globular domain attains its maximal stability. The fact that the negative charges of the phosphates can disrupt this structure again points to their localization in the region important for the stability of the folded domain. That region could be either the folding domain itself or a part of the molecule interacting with it due to some favorable conformation, or both. To discriminate between these possibilities

FIGURE 9: Schematic representation of the structure of *Physarum* histone H1 at high ionic strength with the localization of the irreversible, interphase phosphorylation. The part of C-terminal flank corresponding to 50–70 amino acids forms a loosely folded domain interacting with the trypsin-resistant globular region (open box). The approximate position of interphase phosphorylation is marked by the black box. Note that the positions of mitotic phosphorylations are not shown.

we analyzed the distribution of phosphates (by autoradiography) in the products of limited digestion with trypsin of H1 isolated from $[^{32}P]PO_4$-labeled cultures (Figures 4 and 5). The results of these experiments ruled out the possibility of phosphorylation occurring within the stable, trypsin-resistant peptide of *Physarum* H1, showing it to be free of radioactivity. However, the majority of the label from $[^{32}P]PO_4$ was accumulated within the relatively trypsin-resistant products (named P1 and P2), which we identified as direct precursors of the stable, trypsin-resistant peptide (Figure 6). The results of the analysis of the precursor–product relationship and the distribution of the phosphate label in P1 and P2 are consistent with the interpretation that P1 is a direct precursor of P2 and the latter of the trypsin-resistant peptide. The majority of the phosphates is retained in P2 during its formation from P1. The above analysis and the information that the interphase phosphorylation in 95% concerns the large C-terminal chymotryptic fragment of *Physarum* H1 allowed the conclusion that the P1 and P2 products consist of the trypsin-resistant peptide (the globular domain) and the sequences directly adjacent to its C-terminus.

The relative trypsin resistance of P1 and P2 and the fact that their stability during trypsin digestion increased with increasing ionic strength (Figure 7) suggested that in both products the sequences adjacent to the trypsin-resistant peptide, independently of whether phosphorylated were involved in some kind of folding.

The schematic structure of *Physarum* histone H1 at high ionic strength is shown in Figure 9, which summarizes the interpretation of the obtained results. The scheme postulates that the trypsin-resistant peptide (equivalent to the central globular domain) of *Physarum* H1 interacts with a more loosely folded structure formed from a part of the unusually long C-terminal flank. As judged from the position of P1 product in $NaDodSO_4$ gel electrophoresis, the loosely folded part of the C-terminal flank could amount to 50–70 amino acids. Within this part there is a cluster of phosphorylation sites localized close to the C-terminus of the globular domain, which become irreversibly phosphorylated in the interphase of the mitotic cycle. Such a localization of phosphates would allow a direct influence on the stability of the globular domain. It should be noted that the proposed involvement of a large part of the C-terminal flank in the interactions with the globular domain, provided it occurred also in vivo, could explain why the *Physarum* chromatin has a normal nucleosomal repeat length (Johnson et al., 1976; Staroń et al., 1977) despite the abnormal size of its H1. The remaining, extended part of the C-terminal flank is of a size of C-terminal flanks of typical H1 histones.

The examination of the overall effect of the interphase phosphorylation on the stability of the trypsin-resistant peptide of *Physarum* H1 at different ionic strengths (Figure 8) revealed that the phosphorylation occurring in the C-terminal flank exerted a pronounced stabilizing effect on the folding of the globular domain. This was reflected by the fact that

the stable, trypsin-resistant peptide formed at 300 mM lower NaCl concentration for phosphorylated than for dephosphorylated H1.

What is the physiologic meaning of the stable, interphase phosphorylation of *Physarum* histone H1? The mitotic cycle analysis of the phosphorylation of *Physarum* H1 (Jerzmanowski & Maleszewski, 1985) revealed that H1 is deposited on chromatin DNA in a nonphosphorylated form and is subsequently phosphorylated to the level of 8–12 phosphates per molecule in the mid-G2 phase. This interphase level of phosphorylation remains stable with H1 in the following cycles. Superimposed on it is the cyclic, mitotic superphosphorylation, which increases transiently the total amount of phosphates to more than 20 per molecule of H1 in mitosis (Jerzmanowski & Maleszewski, 1985; Mueller et al., 1985). The main difference between the interphase phosphorylation of *Physarum* and mammalian H1s is that in *Physarum* this event is irreversible and concerns not a fraction but 100% of H1 molecules. It should be noted that the interphase phosphorylation of *Physarum* H1 is delayed with respect to the deposition of the newly synthesized H1 on the chromatin DNA. In particular in the late S phase when the majority of DNA is already replicated and complexed with histones, about half of the total H1 (i.e., the new H1) is still in the nonphosphorylated form (Jerzmanowski & Maleszewski, 1985). The fact that in the DNase I released fraction of *Physarum* chromatin, which contains DNA of the average size of 20–40 kilobases (Czupryn & Toczko, 1985), only the nonphosphorylated H1 has been found (Figure 1, lower panel) indicates a nonrandom distribution of at least part of newly synthesized H1. If the decreased ability to form a folded domain at lower ionic strength by dephosphorylated *Physarum* H1 in solution reflected its decreased ability to condense chromatin in vivo, such a distribution could have a profound functional significance. In this context it is of interest that in *Physarum*, which lacks the G1 phase, the peak of transcription of a poly(A)+ RNA has been correlated with the S phase of the mitotic cycle (Melera, 1980).

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