A Phosphorylation-Induced Major Structural Change in the N-Terminal Domain of the P Protein of Chandipura Virus†

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ABSTRACT: It has previously been shown that phosphorylation of P protein of vesicular stomatitis virus as well as Chandipura (CHP) virus is required for transcription activation and replication switch. The structural nature of this crucial conformational change, however, is largely unknown. We have studied the phosphorylation-associated conformational change in the P protein of Chandipura (CHP) virus using chemical modification, fluorescence, and circular dichroism spectroscopy. Sulfhydryl groups of unphosphorylated CHP-P protein are unreactive to DTNB under nondenaturing conditions. Upon phosphorylation, one sulfhydryl group becomes reactive. We have identified this sulfhydryl group as cysteine 57. The two tryptophan residues (105 and 135) become significantly more buried in the phosphorylated protein. Circular dichroism spectra show significant enhancement in the far-UV region upon phosphorylation. Anisotropy decay of AEDANS-labeled C57 CHP-P protein shows rapid rotation of the probe, suggesting significant mobility of the N-terminal domain in the phosphorylated P protein. The results suggest a global conformational change in the N-terminal domain of the P protein is induced by phosphorylation and yet the phosphorylated N-terminal domain shows significant flexibility.

Regulation of transcription is one of the most important areas of molecular biology. Diverse strategies have evolved in various organisms in response to different requirements. These diverse strategies of regulation have to be studied in order to gain insight into the mechanism of control. Most of the current work has focused on DNA-dependent transcription systems, primarily because of its widespread occurrence. RNA-dependent transcription systems occur in some RNA viruses, and recent studies have suggested that activating transcription factors are an essential component of the viral life cycle in the rhabdovirus family (*1*).

This system is most well characterized in the vesicular stomatitis virus (VSV) and the related Chandipura virus. The RNA genomes of these viruses are transcribed and replicated by an RNA-dependent RNA polymerase, packaged with the virion. The RNA-dependent RNA polymerase has two components, a 241 kDa protein called L and a smaller phosphoprotein (33 kDa) called P. The P protein in these systems acts as a transcription activator, which helps the RNA-dependent RNA polymerase to transcribe the 11 kb long genome (*2*). The P protein has an acidic amino-terminal region that is crucial for its activation function (*3*). It has been demonstrated that the VSV New Jersey [VSV(NJ)] P protein exists in three different forms: unphosphorylated, singly phosphorylated (P1), and doubly phosphorylated (P2). The first phosphorylation is carried out by the host casein kinase II (CKII) and the second one by the L-associated kinase. Using bacterial expression systems to obtain unphosphorylated protein, it has been demonstrated that phosphorylation acts as a switch to activate protein P, the transcription factor (*4*, *5*). In the Chandipura virus, however, only one serine phosphorylation takes place (*6*). In both cases the phosphorylations take place in the N-terminal acidic region. Recent reports suggest that in the VSV(NJ), the phosphorylation is accompanied by a significant change in the CD spectrum of the protein as well as in the Stokes radius as measured by size-exclusion chromatography (*7*). The nature of the phosphorylation-dependent conformational change is unknown at the present time.

Recent reports from Lenard and co-workers suggests that the role of phosphorylation may be different in related VSV(IND) (*8*). In contrast to the VSV(NJ) and Chandipura, the unphosphorylated protein is partially active in vitro, and phosphorylation appears to cause no significant change in the circular dichroism spectrum of the protein. Clearly, an understanding of the nature of the phosphorylation-dependent conformational change is a prerequisite for elucidation of the mechanism of transcription activation in RNA-dependent transcription systems as well as the understanding of apparently different strategies used by related viruses of the same family. In a broader context, phosphorylation-dependent transcription regulation is one of the fundamental ways in which many organisms respond to environmental stimuli, and hence uncovering the structural basis of such regulation has wider implications, far beyond the rhabdoviral family. In this article, we have explored the nature of the phosphorylation-dependent conformational change in the P protein

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of Chandipura virus using chemical modification, fluorescence, and CD spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. Q-Sepharose was from Pharmacia United (Sweden). Caesin kinase II was purchased from Boehringer Manneheim (Germany). DTNB[1] was purchased from Sigma Chemical Co. (St. Louis, MO). ANS was from Molecular Probes Inc. (Eugene, OR).

Bacterial Strains and Plasmids. E. coli BL21(DE3) was used to express P protein from pET3apc plasmid for the purification of P protein (*9*).

Expression of P Protein and Purification. The P protein was solubilized either by denaturation−renaturation from inclusion bodies or from the soluble fractions directly. Protein purified by either method gave identical results.

The Denaturation−Renaturation Method. The pET3aPC clone was transformed in BL21(DE3) (10) for the production of P protein. BL21(DE3) containing plasmid pET3aPC was grown in Luria broth supplanted with 0.3% glucose and 100 mg/mL ampicillin to an OD of 0.3. It was then induced with 0.5 mM IPTG (final concentration) and further grown for a period of 2 h. The cells were then harvested and washed with 50 mM Tris-HCl, pH 8.0, followed by lysis with lysozyme (0.1 mg/g of cell). After washing with the same buffer containing 1 M NaCl, the protein in the inclusion body was solubilized with 8 M urea in 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 0.1% Tr-X-100. The protein dissolved in urea was then dialyzed against non-urea-containing buffer.

Isolation from Soluble Fraction. BL21(DE3) was transformed by petPC DNA in LA containing ampicillin (100 μg/mL) at 25 °C. After overnight growth, the colonies were scraped to LB containing 100 μg/mL ampicillin, 20 mM glucose and grown to an OD of 1 at 25 °C. After induction with 0.5 mM IPTG (final concentration), the culture was grown for a further 6 h. The cells were centrifuged and washed with 50 mM Tris-HCl, pH 8.0. The cells were then lysed with 20 μg/mL lysozyme in the presence of DNase I and centrifuged, and the supernatant was collected. It was found that about 60% of the protein became soluble by this method.

The P protein was purified to homogeneity by anion exchange chromatography with Q-Sepharose using a sodium chloride gradient. The protein elutes between 300 and 400 mM NaCl. All protein concentrations were measured at 280 nm using a double beam spectrophotometer. The molar extinction coefficient used was 20 000 M^{-1} cm^{-1} (*6*).

In Vitro Kinasing and Purification of P Protein. The purified P protein was phosphorylated in vitro, using human recombinant caesin kinase II in 20 mM MES buffer, pH 6.9, containing 130 mM KCl, 10 mM $MgCl_2$, and 4.8 mM dithiothreitol. The reaction was conducted at 37 °C with 50 μM ATP. The mixture was then separated on a Sephacryl S-300 column equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The unphosphorylated protein elutes at around 32 kDa, whereas the phosphorylated protein elutes at around 65 kDa (*6*).

Determination of Sulfhydryl Content by DTNB Titration. Aliquots of 5 μM each of phosphorylated and unphosphorylated P protein were incubated with 0.5 mM DTNB in 50 mM Tris-HCl, pH 8.0 at 25 °C. The reaction was monitored at 412 nm in a Hitachi UV2000 spectrophotometer after appropriate base line correction. A molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} was used to calculate the number of sulfhydryl groups reacted.

Fluorescence Spectroscopy. Steady-state fluorescence spectra were recorded in a Hitachi F3010 spectrofluorometer, equipped with a spectra addition and subtraction facility. The fluorescence experiments were carried out in a thermostated chamber where the temperature was maintained at 25 °C by circulating water. The band-passes of both the excitation and emission were 5 nm, unless stated otherwise. All fluorescence experiments were carried out in a 1 cm path length cuvette.

Acrylamide Quenching of Tryptophan Fluorescence. Bacterially expressed P protein in 50 mM Tris-HCl, pH 8.0, was titrated with increasing concentrations of acrylamide, and fluorescence intensity values were determined. The excitation wavelength was 295 nm, and the emission was monitored at 340 nm. A blank titration was conducted in which 50 mM Tris-HCl buffer, pH 8.0, was titrated with acrylamide and the fluorescence values were subtracted from the corresponding observed protein fluorescence values. The intensity values were corrected for the inner filter effect. The fluorescence intensity values were plotted either as a Stern−Volmer plot:

$$F_o/F = 1 + K_{sv}[Q] = 1 + k_q\tau_o[Q]$$

where F_o is the fluorescence intensity in the absence of the quencher, F is the fluorescence in the presence of the quencher, K_{sv} is the Stern−Volmer constant, k_q is the bimolecular quenching constant, τ_o is the lifetime in the absence of the quencher, and [Q] is the quencher concentration, or as a Lehrer plot:

$$F_o/(F_o - F) = 1/f_a + 1/f_aK_{sv}[Q]$$

where f_a is the fractional contribution which is quenched by low quencher concentrations, to the total fluorescence (*11*).

ANS Binding. Both purified phosphorylated and unphosphorylated P proteins at a concentration of 5 μM in 50 mM Tris-HCl, pH 8.0, were titrated with increasing concentrations of ANS at 25 °C. Fluorescence intensities were measured with the excitation wavelength at 390 nm and the emission wavelength at 482 nm.

Labeling of Phosphorylated Protein with IAEDANS. The purified phosphorylated protein (100 μM) was reacted with 5 mM IAEDANS at 37 °C for 2 h. The protein was separated from the unreacted IAEDANS on a Sephadex G-25 column equilibrated with 50 mM Tris-HCl buffer, pH 8.0.

Time-Resolved Fluorescence Intensity and Anisotropy. Time-resolved fluorescence measurements were made in an APL SP-70 D (Applied Photophysics, U.K.) lamp based nanosecond fluorescence spectrometer which used a time-correlated single photon counting technique. The L-geometry and toggling method was used to measure anisotropy decay. This method uses a motor-driven polarizer to toggle between parallel and crossed emission polarizer orientations, accumulating data into separate memory segments of the multichannel analyzer. The data were analyzed using the

[1] Abbreviations: CHP, Chandipura; IAEDANS, 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid; ANS, 1-anilino-8-naphthalenesulfonic acid; DTNB, dithiobis(2-nitrobenzoic acid).

direct vector reconvolution program provided by Applied Photophysics. Excitation was done at 337 nm, and the emission was set at 520 nm. The measurements were done in 50 mM Tris-HCl buffer, pH 8.0, at ambient temperature, which was 25 ± 1 °C. For tryptophan lifetime measurements, pulsed laser sources were used.

Circular Dichroism. Circular dichroism spectra were measured in a JASCO J-600 spectropolarimeter. The measurements were conducted in 50 mM Tris-HCl, pH 8.0, at ambient temperature, which was 25 ± 1 °C. The measurements were done with a band width of 1.0 nm and a scan speed of 50 nm/min; a 1 mm path length cuvette was used throughout. Spectra of unphosphorylated and phosphorylated proteins were taken by different methods. For unphosphorylated protein, the spectrum of the buffer was subtracted to correct for the background. Another way of measuring the unphosphorylated P protein spectra was to incubate the P protein with dATP and casein kinase II and subtract the spectra of reactions with identical components except the P protein (dATP is not a substrate of casein kinase II). For obtaining the spectra of the phosphorylated P protein, the unphosphorylated protein was phosphorylated with casein kinase II as described and spectra were taken. A mixture of all components except the P protein was incubated for the same length of time, and the spectra of the mixture were subtracted from the phosphorylated P protein spectra. In other cases, the phosphorylated P protein was purified by Sephacryl S-300 column chromatography, and then the spectrum was measured. The spectra of phosphorylated and unphosphorylated P protein were consistent when measured by different methods. The protein concentrations were 5 μM. Secondary structure contents were estimated by a neural network based model.

RESULTS

Phosphorylation of P protein of Chandipura virus is essential for the transcription of the virus in vitro (*6*). It is possible that the activation involves a phosphorylation-induced conformational change. One of the best ways to study conformational changes in proteins is by fluorescence spectroscopy. Many covalent and noncovalent environment-sensitive fluorescence probes have been used in recent years for this purpose. Sulfhydryl groups are often chosen as suitable attachment points for covalent external probes because of the high specificity of modification of the sulfhydryl groups and limited numbers present in the proteins. The CHP−P protein has five sulfhydryl groups at positions 37, 57, 149, 172, and 286. Figure 1 shows the reaction of DTNB with phosphorylated and unphosphorylated P protein. The unphosphorylated protein shows virtually no reactivity, whereas the phosphorylated protein shows a slow increase of absorbance which in the limit approaches one reacted sulfhydryl group per subunit. Since this sulfhydryl group is unreactive in the unphosphorylated protein, which is a monomer, and reactive in the phosphorylated protein, which is a dimer (*6*), a phosphorylation-induced conformational change is likely the cause of this changed reactivity. Evidence now points toward serine 62 as the phosphorylation site both in vitro and in vivo (*6*). We have constructed mutant S62A, and as a control, this mutant protein was incubated under the same phosphorylation conditions and then reacted with DTNB. As shown in Table 1, the extent of reaction is

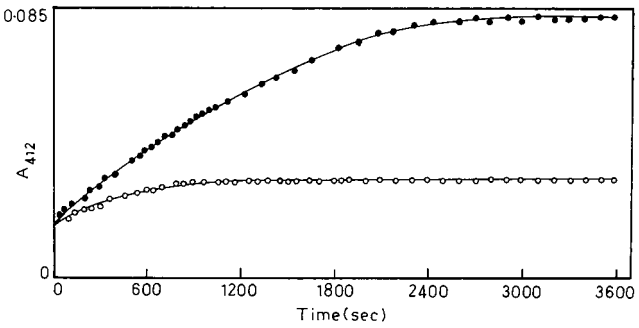

FIGURE 1: Time course of reaction of sulfhydryl groups of unphosphorylated (○) and phosphorylated P (●) protein with 0.5 mM DTNB. Both proteins were incubated at 5 μM concentration with 0.5 mM DTNB in 50 mM Tris-HCl, pH 8.0, at 25 °C, and the reaction was monitored by measuring the absorbance at 412 nm.

Table 1: DTNB Reactivities of Mutant and IAEDANS-Modified P Proteins

	A_{412}		
time (s)	AEDANS-P1	P0	S62A
0	0.016	0.015	0.017
600	0.026	0.024	0.023
1200	0.032	0.031	0.029
1800	0.03	0.032	0.032
2400	0.031	0.031	0.031
3000	0.031	0.031	0.031
3600	0.031	0.031	0.031

like that of the unphosphorylated protein (P0), suggesting that phosphorylation has a causal relationship with increased sulfhydryl reactivity.

Since several sulfhydryl groups are present in the CHP−P protein, we have attempted to locate this reactive sulfhydryl group. The sequence suggests that tryptic peptide 43−102 is by far the largest of the tryptic peptides and contains the phosphorylation site. When exhaustively digested with trypsin, phosphorylated P protein produces many small peptides, out of which the largest one has an approximate molecular mass of 6 kDa. This band contains all the ^{32}P label when ^{32}P-labeled CHP−P protein is exhaustively digested with trypsin [for a picture of the gel, please refer to Figure 3(b) of (*6*)]. This band then originates from peptide 43−102 of the protein and contains the phosphorylation site. The reactive sulfhydryl group in the phosphorylated P protein can be labeled with IAEDANS, and approximately 1 mol of AEDANS was incorporated per mole of protein monomer (see Experimental Procedures). To locate the sulfhydryl group, we have digested phosphorylated IAEDANS-labeled CHP−P protein with trypsin and separated the peptides on SDS−PAGE. A phosphorylated native CHP−P trypsin digest and standard molecular mass markers were run as a control to locate the bands. Bands corresponding to the 6 kDa peptide and those corresponding to peptides of molecular mass lower and higher than 6 kDa were excised and eluted, and then fluorescence was measured. Table 2 shows the results of such fluorescence measurements. Most of the AEDANS fluorescence is located in the 6 kDa and native bands, indicating that the reactive sulfhydryl group is within the 6 kDa peptide. The only cysteine residue present in this peptide is C57, suggesting that the reactive cysteine is C57. To demonstrate that IAEDANS is reacting with the sulfhydryl group and not with other nucleophilic groups in the protein, we have measured the reaction of AEDANS-CHP-P protein with

Table 2: Fluorescence Intensity of Different Gel-Eluted Fractions of AEDANS-Labeled CHP-P Protein after Trypsin Digestion[a]

eluted gel region	fluorescence intensity
combined gel region lower than 6 kDa	40
gel region corresponding to 6 kDa	246.1
gel region above 6 kDa up to the native protein band	44.8
gel region corresponding to the native protein	111.0

[a] Phosphorylated IAEDANS-labeled P protein was digested exhaustively with 20 μg/mL TPCK-trypsin in 50 mM Tris-HCl, pH 8.0, and separated in 15% SDS−PAGE. Different regions of the gel were excised, washed with distilled water, crushed in 1 mL of 50 mM Tris-HCl, pH 7.9, containing 5 mM DTT and 150 mM NaCl, and incubated for 8 h at 25 °C. After centrifugation, the supernatant was precipitated with 4 volumes of ice-cold acetone, washed with 80% acetone, dried, and dissolved in 50 mM Tris-HCl, pH 8.0.

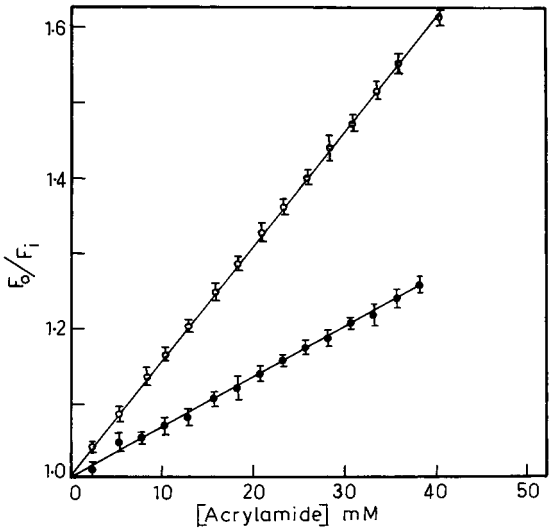

FIGURE 2: Stern−Volmer plot of acrylamide quenching of tryptophan fluorescence of unphosphorylated (○) and phosphorylated (●) P protein. The solution conditions were 50 mM Tris-HCl, pH 8.0, at 25 °C. The excitation and emission wavelengths were 295 and 340 nm, respectively. On the *y*-axis, F_0 stands for the fluorescence value without any acrylamide, and F_i stands for the fluorescence value after addition of acrylamide at each data point.

DTNB under the conditions described above. The results of this reaction are shown in Table 1. The sulfhydryl reactivity is identical to unphosphorylated protein and the S62A mutant, suggesting that the reactive sulfhydryl group is blocked upon derivatization of 1 mol per mole of AEDANS. This suggests that C57 is the attachment site of the AEDANS probe.

The CHP-P protein has two tryptophan residues, W105 and W135. Tryptophans are often a good probe for studying protein conformational changes. In many multi-tryptophan proteins, a change in environment, and consequently the fluorescence of one tryptophan residue, is often obscured by the fluorescence of other tryptophan residues leading to small or insignificant overall changes. In some cases, acrylamide quenching can be used to resolve tryptophan fluorescence into components based on the accessibility of tryptophans to collisional quenchers, such as acrylamide (*12*). Figure 2 shows the Stern−Volmer plot of acrylamide quenching of tryptophan fluorescence of unphosphorylated and phosphorylated CHP−P protein. The plots are linear up to 40 mM acrylamide (highest concentration used), indicating the absence of any significant static quenching component. The tryptophan fluorescence of the unphosphorylated protein is fairly quenchable with a K_{sv} of 16.6 M^{-1}. The tryptophan fluorescence of phosphorylated protein, in contrast, is much less quenchable, with a K_{sv} of 6.5 M^{-1}. The time-resolved decay of the tryptophan fluorescence can be well described by a double exponential (data not shown). The unphosphorylated and the phosphorylated proteins have lifetimes of 1.23 ns (amplitude = 0.42)/4.7 ns (amplitude = 0.58) and 1.39 (amplitude = 0.415)/ 4.74 (amplitude = 0.585) ns, respectively. The calculated second-order average lifetimes are 4.14 and 4.17 ns for the unphosphorylated and phosphorylated CHP−P protein, respectively. The quenching constants, K_q, calculated from the lifetime data and Stern−Volmer constants, are 4×10^9 M^{-1} s^{-1} and 1.55×10^9 M^{-1} s^{-1} for the unphosphorylated and phosphorylated CHP−P protein, respectively. This suggests that, on average, tryptophans of the phosphorylated protein are less accessible to collisional quenchers. The tryptophan fluorescence emission maximum of the unphosphorylated form is 344.2 nm, whereas that of the phosphorylated form is 341.2 nm. This additional blue shift upon phosphorylation suggests that one or more tryptophans shift to a more apolar environment upon phosphorylation. This is consistent with the decrease of accessibility of one or more tryptophans upon phosphorylation as described above.

The Lehrer plot, a reciprocal version of the Stern−Volmer plot, has often been used to resolve the contribution of different tryptophans in the overall fluorescence spectrum of proteins (*12*). In this plot ($F_0/\Delta F$ vs $1/Q$), the *y*-axis intercept of the extrapolation of the plot at low quencher concentrations is a reflection of the fraction of total fluorescence that is quenchable with low concentrations of acrylamide. Thus, the nonlinearity or intercept values higher than 1 reflect different quenching constants of different tryptophans. Lehrer plots of the unphosphorylated and the phosphorylated CHP−P protein are linear, and both pass through 1 although with different slopes, indicating that the quenching constants of both tryptophans are similar in a given state (data not shown). This rough equivalence with respect to the quenching constant of the two tryptophans is preserved in both the unphosphorylated state and the phosphorylated state. The lower bimolecular quenching constant k_q in the phosphorylated state, however, indicates that both tryptophans in the phosphorylated protein are less accessible to collisional quenchers such as acrylamide. Since both tryptophans are situated in the N-terminal half of the protein, this implies a phosphorylation-induced conformational change in the N-terminal half of the protein. This, however, does not exclude the possibility of a conformational change in the C-terminal half as well.

Circular dichroism spectroscopy has been widely used as a sensitive monitor of secondary structure in proteins. We have attempted to see if phosphorylation-induced conformational change is accompanied by changes in the secondary structure. Figure 3 shows far-UV circular dichroism spectra of phosphorylated and unphosphorylated CHP−P protein. The protein shows 10−15% enhancement of CD intensity (negative) upon phosphorylation. A similar enhancement was observed by Das et al. (*7*) on the homologous P protein of VSV(NJ). The CD spectra were used to deduce the secondary structure composition. It appears from the analysis that there is a significant increase in the fraction of α-helix in the phosphorylated spectrum, with a concomitant decrease of

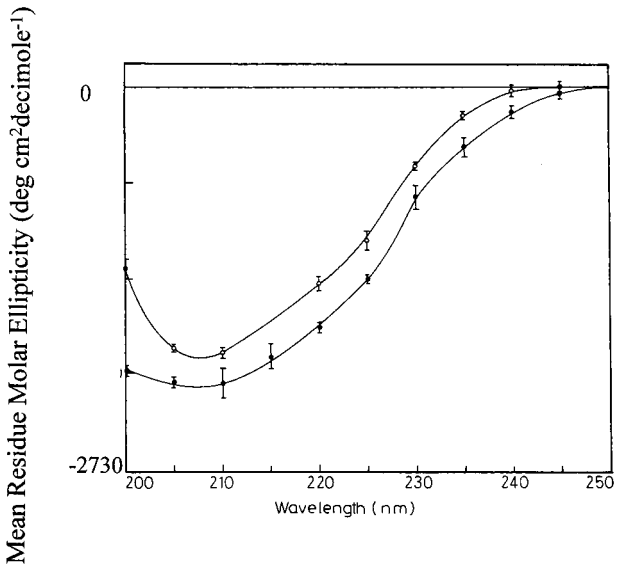

FIGURE 3: Far-UV CD spectra of the unphosphorylated (○) and phosphorylated (●) P protein. The experiments were conducted in 50 mM Tris-HCl, pH 8.0, at 25 °C. The bandwidth was 1.0 nm, and the scan speed was 50 nm/min. Ten spectra were averaged to improve the signal-to-noise ratio. Four different experiments were averaged to yield the error bars, which are shown only at 5 nm intervals. The protein concentrations were 5 μM.

other secondary structure elements. Thus, the phosphorylation causes a significant increase of the helix content at the expense of both coil and sheet. This is qualitatively similar to that observed for VSV(NJ) P protein (*7*). This implies a significant change of secondary structure upon phosphorylation.

Anilinonaphthalenesulfonates, such as ANS, bis-ANS, and PRODAN, have emerged as major noncovalent fluorescence probes in protein structure−function studies (*13*, *14*). They bind to accessible hydrophobic sites of proteins and act as a sensitive monitor of change in the accessible hydrophobic surface area. In cases where there is a large change in the accessible hydrophobic surface area, such as in the native to molten globule transition, much increased binding of ANS is seen (*15*). To see whether the major conformational change induced by phosphorylation is accompanied by a significant change in the accessible hydrophobic surface area, we have studied ANS binding to unphosphorylated and phosphorylated CHP−P protein. Figure 4 shows the ANS binding isotherm of unphosphorylated and phosphorylated CHP−P proteins. ANS binding to both the unphosphorylated and the phosphorylated form is similar at lower concentrations of ANS, but a significant decrease of ANS binding to phosphorylated CHP−P protein is seen at higher ANS concentrations. Interactions of anilinonaphthalenesulfonates with proteins are complex. Bis-ANS in particular is known to have many binding sites, and similar behavior is known for ANS (*14*, *16*). The binding isotherms shown here can be interpreted as a result of ANS binding to several sites, and phosphorylation causes a significant decrease or abolition of binding to the weaker site(s). This is consistent with some decrease of the accessible hydrophobic surface area.

Since both the reactivity of cysteine 57 and the environments of the two tryptophans are affected by the phosphorylation-induced conformational change, knowledge of distances between C57 and the tryptophans may shed light on

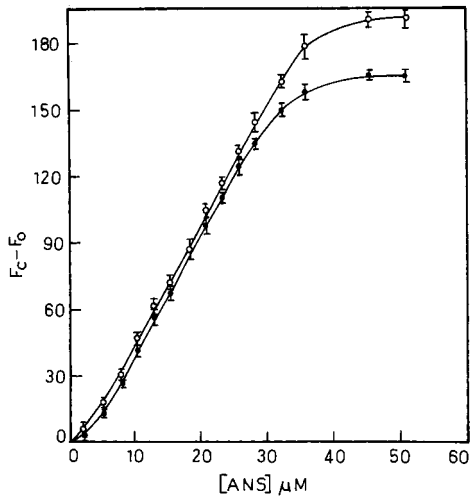

FIGURE 4: Titration of unphosphorylated (○) and phosphorylated (●) P protein with ANS. The experiments were conducted in 50 mM Tris-HCl, pH 8.0, at 25 °C. The excitation and emission wavelengths were 390 and 482 nm, respectively. On the *y*-axis, F_c represents the fluorescence value of ANS−protein complexes, whereas F_o represents the fluorescence of the same concentration of ANS in buffer.

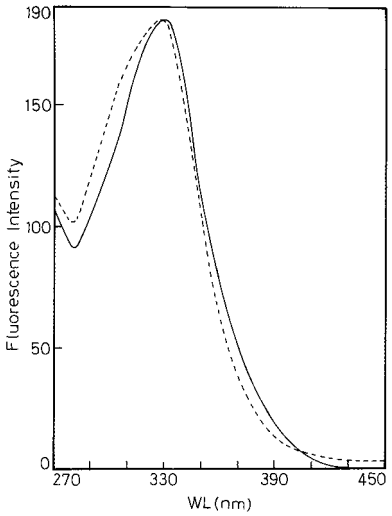

FIGURE 5: Fluorescence energy transfer experiments of IAEDANS-labeled phosphorylated P protein with tryptophans (- - -). The emission was set at 520 nm, and the excitation was scanned from 270 to 450 nm. For comparison, the cysteine−IAEDANS conjugate excitation spectrum is shown under the same conditions (—). The experiments were conducted in 50 mM Tris-HCl, pH 8.0, at 25 °C.

the global nature of the conformational change. Figure 5 shows the fluorescence energy transfer experiment from the AEDANS-labeled C57 to the two tryptophans W105 and W135. We have compared excitation spectra of AEDANS-labeled P protein with the excitation spectra of the cysteine conjugate of AEDANS. If any energy transfer occurs, it would lead to enhanced fluorescence at around 280−295 nm (tryptophan absorbance band) when compared to the cysteine conjugate. As can be seen from the figure, two excitation spectra are similar, indicating a low degree of energy transfer between the tryptophans and the C57−AEDANS. The R_o of tryptophan to AEDANS energy transfer is calculated to be 27.5 Å (*17*) in this protein. The calculated energy transfer efficiency is 0.19, which corresponds to a minimum distance of 35 Å (*18*). If energy transfer from only one tryptophan

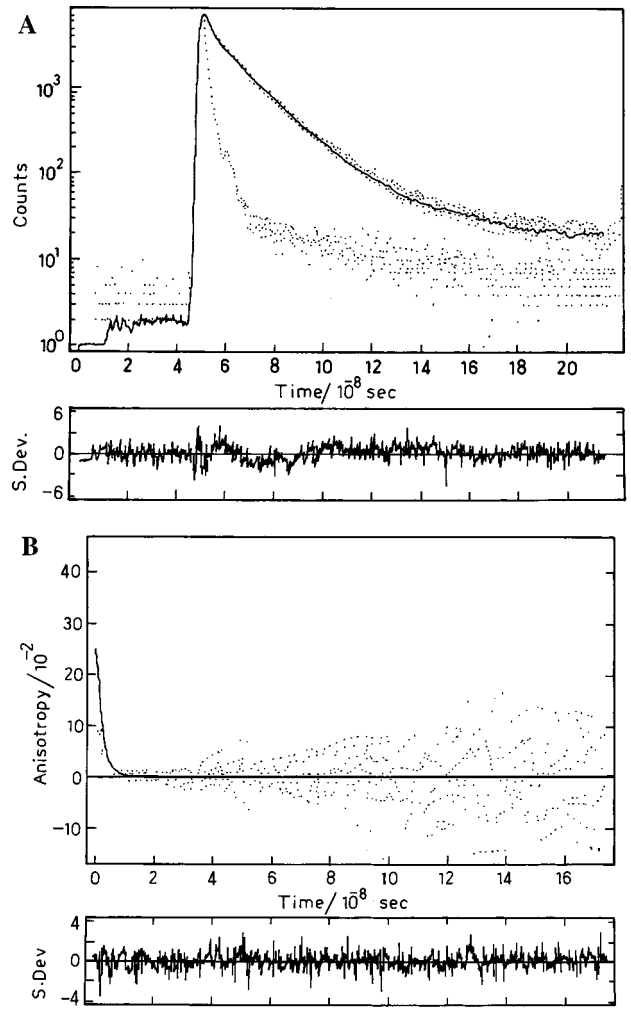

FIGURE 6: Decay of (A) fluorescence intensity and (B) fluorescence anisotropy of IAEDANS-labeled phosphorylated P protein. The *y*-axis represents the number of photons per channel in (A) and anisotropy values in (B) after conversion of the collected intensity data with parallel and perpendicular emission polarizers. The excitation was at 337 nm, and the emission was monitored at 520 nm. The experiments were conducted in 50 mM Tris-HCl, pH 8.0, at 25 °C.

occurs, one tryptophan is about 35 Å away from the AEDANS−C57 and the distance is much greater for the other tryptophan. If energy transfer occurs from both tryptophans, the distance from both tryptophans would be longer than 35 Å. This suggests that C57 is probably at least 35 Å away from both tryptophans. The lack of energy transfer could also be due to unfavorable values of κ^2. However, as we show later, the AEDANS label is highly mobile, and hence κ^2 is likely to be around its random orientation value of 2/3.

The phosphorylation-induced conformational change appears to introduce a major restructuring of the N-terminal part of CHP−P protein. There are many cases known where ligand binding or modification leads to disorder−order transitions (*19*). Such a transition may lead to compaction and loss of mobility. We have attempted to measure the flexibility of the N-terminal part of the CHP−P protein by measuring time-resolved anisotropy decay of C57−AEDANS-labeled phosphorylated P protein. Figure 6 shows the fluorescence intensity and anisotropy decays of AEDANS-labeled CHP−P protein. Fluorescence intensity decay can be fitted to two components having lifetimes of 1.35 and 14.7 ns. These lifetime values are similar to those of other protein−AEDANS conjugates. The anisotropy decay, however, gave a very rapid decay with most of the anisotropy decayed by 10 ns, suggesting a correlation time of considerably less than 10 ns. If spherical, the dimer of CHP−P protein is expected to have a rotational correlation time of 26 ns (*18*). The lower than expected correlation time indicates rapid segmental motion of the AEDANS molecule. This mobility of the probe is unlikely to be due to the flexibility of the probe alone as other AEDANS-labeled proteins show much slower rotational relaxation (*20*). The steady-state anisotropy value of the IAEDANS-labeled protein (at C57) is 0.07 (compared to 0.028 for the IAEDANS alone). Such a low value, when compared with much higher values obtained in other proteins (*21*), supports the conclusion of the flexible nature of the N-terminal domain obtained by anisotropy decay experiments.

DISCUSSION

The P proteins of VSV and related viruses are known to play a crucial role in transcription regulation. The single phosphorylation at Ser62 in CHP−P protein converts the protein into a potent transcription activator and may also be involved in the transcription−replication−transcription switch, which ultimately allows viral multiplication (*3*, *22*). The phosphorylation is known to affect the conformation of the P protein of the related VSV(NJ), although the precise nature of the conformational transition is not well understood (*7*).

In this article, we have investigated the nature of this conformational transition using various spectroscopic and chemical probes. Change in the sulfhydryl reactivity of an N-terminal cysteine residue and a major change in the quenchabilities of two tryptophan residues located at or near the N-terminal domain suggest a major conformational change involving the N-terminal domain. Since C57 and the two tryptophan residues are situated at least 35 Å away, as measured by fluorescence energy transfer, and both are affected by the phosphorylation-induced conformational change, it is likely that the conformational change involves at least a significant part of the N-terminal domain. Major enhancement of the far-UV circular dichroism spectrum suggests a major restructuring of the domain, as well. Reduced binding of ANS suggests a small but significant reduction in the accessible hydrophobic surface area. Since both the tryptophan residues go from a fairly solvent-exposed state to a more buried state and the far-UV CD spectrum is enhanced and is accompanied by a decrease in the accessible hydrophobic surface area, it is likely that the phosphorylation-induced structural change may involve a disorder−order transition. Large estimated distances between the AEDANS probe and the two tryptophans in the phosphorylated protein suggest that the restructured N-terminal domain is not a compact globular structure, interacting tightly with the C-terminal domain. The anisotropy decay of the AEDANS probe suggests a correlation time on the order of 5 ns or lower. This is consistent with a highly mobile and perhaps extended structure of the N-terminal domain in the phosphorylated state.

The lack of reactivity of sulfhydryl groups (37 and 57) located in the N-terminal domain as well as k_q values and

emission maxima of the N-terminal tryptophans (which are significantly less than the completely exposed tryptophans) suggest that the unphosphorylated N-terminal domain may not be completely devoid of structure. In the absence of a specific structural probe in the unphosphorylated protein, it is difficult to speculate about the structure and dynamics of the region.

A number of eukaryotic transcription factors are known to possess stretches of acidic amino acids. It has been shown that tandem repeats of the acidic 11 amino acid stretch are sufficient for transcription activation (*23*, *24*). It has also been suggested that multiple contacts with transcription machinery may be the underlying requirement for such tandem repeats. The CHP−P protein contains a stretch of acidic residues around the phosphorylated serine 62, which may be construed as such a tandem repeat (residues 49−69). Flexibility is of utmost importance if multiple contacts are to be made. It has also been suggested that the unstructured acidic domains may become further structured upon contact with the target molecules (*25*). The gain of structure in the N-terminal domain, seen upon phosphorylation, may be a step in that direction, facilitating the final "induced fit".

Recently, Gao et al. (*8*) have reported that no significant change in the circular dichroism spectra occurs in the phosphorylated and unphosphorylated forms of the P protein of VSV(IND). This protein also forms trimers upon phosphorylation. As pointed out before, the unphosphorylated P proteins in this system (carrying a serine to alanine mutation at the phosphorylation sites) are partially active. This clearly suggests that different members of the family may respond to phosphorylation in a fundamentally different way. Why such fundamentally different strategies have evolved in different members of the same family of the virus remains to be elucidated.

REFERENCES

1. Banerjee, A. K. (1987) *Microbiol. Rev. 51*, 66−87.
2. Emerson, S. U. (1987) in *Rhabdoviruses* (Wagner, R. R., Ed.) pp 75−128, Plenum Press, New York.
3. Chattopadhyay, D., and Banerjee, A. K. (1987) *Cell 49*, 407−414.
4. Barik, S., and Banerjee, A. K. (1992) *J. Virol. 66*, 1109−1118.
5. Barik, S., and Banerjee, A. K. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6570−6574.
6. Chattopadhyay, D., Raha, T., and Chattopadhyay, D. (1997) *Virology 239*, 11−19.
7. Das, T., Gupta, A. K., Sims, P. W., Gelfand, C. A., Jentoft, J. E., and Banerjee, A. K. (1995) *J. Biol. Chem. 270*, 24100−24107.
8. Gao, Y., Greenfield, N. J., Cleverley, D. Z., and Lenard, J. (1996) *Biochemistry 35*, 14569−14573.
9. Chattopadhyay, D., and Chattopadhyay, D. (1994) *Cell. Mol. Biol. Res. 40*, 693−698.
10. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
11. Lehrer, S. S. (1971) *Biochemistry 10*, 3254−3263.
12. Bandyopadhyay, S., Banik, U., Bhattacharyya, B., Mandal, N. C., and Roy, S. (1995) *Biochemistry 34*, 5090−5097.
13. Saha, R., Banik, U., Bandopadhyay, S., Mandal, N. C., Bhattacharyya, B., and Roy, S. (1992) *J. Biol. Chem. 267*, 5862−5867.
14. Shi, L., Palleros, D. R., and Fink, A. L. (1994) *Biochemistry 33*, 7536−7546.
15. Das, B. K., Bhattacharyya, T., and Roy, S. (1995) *Biochemistry 34*, 5242−5247.
16. Masui, R., and Kuramitsu, S. (1998) *Biochemistry 37*, 12133−12143.
17. Bhattacharyya, A., Bhattacharyya, B., and Roy, S. (1993) *Eur. J. Biochem. 216*, 757−761.
18. Cantor, C. C., and Schimmel, P. R. (1980) *Biophysical Chemistry*, Vol. II, W. H. Freeman, San Fransisco.
19. Spolar, R. S., and Record, M. T., Jr. (1994) *Science 263*, 777−784.
20. Suzuki, S., Kawato, S., Kouyama, T., Kinosita, K., Ikegami, A., and Kawakita, M. (1989) *Biochemistry 28*, 7734−7740.
21. Morita, J., Takashi, R., and Ikebe, M. (1991) *Biochemistry 30*, 9539−9545.
22. Takacs, A. M., Barik, S., Das, T., and Banerjee, A. K. (1992) *J. Virol. 66*, 5842−5848.
23. Seipel, K., Georgiev, O., and Schaffner, W. (1994) *Biol. Chem. Hoppe-Seyler 375*, 463−470.
24. Blair, W. S., Bogerd, H. P., Madore, S. J., and Cullen, B. R. (1994) *Mol. Cell. Biol. 14*, 7226−7234.
25. Schmitz, M. L., dos Santos Silva, M. A., Altmann, H., Czisch, M., Holak, T. A., and Baeuerle, P. A. (1994) *J. Biol. Chem. 269*, 25613−25620.

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