The Transcriptional Form of the Phosphoprotein of Vesicular Stomatitis Virus Is a Trimer: Structure and Stability

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ABSTRACT: The phosphoprotein (P) of vesicular stomatitis virus was previously shown to assemble into a homomultimer upon phosphorylation by casein kinase II. It thus acquired transcriptional activity, including the ability to bind to the other two transcriptional components, the polymerase L and the N-RNA template. This multimer has now been found to be a trimer using a His-tag dilution method. Trimer stability was assessed using a variation of this method, by measuring the rate of exchange of monomers between preformed tagged and untagged trimers at different values of pH and ionic strength. Exchange rates increased with increasing ionic strength and were similar at pH 6, 8, and 10, but the trimer was completely dissociated at pH 4. This suggests that the trimer is stabilized by electrostatic interactions, probably involving carboxylate and guanidino groups. Addition of viral L protein stabilized the P trimers, completely preventing subunit exchange under transcription conditions. The association constants (K_{ass}) for trimerization of partially active D and A substitution mutants were also determined by His-tag dilution and found to correlate well with transcriptional activity, further confirming that the active species is the trimer. Circular dichroism spectra were identical for phosphorylated and unphosphorylated wild-type P protein and for D and A mutants known to be predominantly trimeric and monomeric, respectively.

Vesicular stomatitis virus (VSV) is a small, nonsegmented, negative strand RNA virus that transcribes and replicates its genome entirely in the cell cytoplasm. A fully active transcription complex (the nucleocapsid) of VSV is composed of three distinct, separable components: the N-RNA template, consisting of the 11 kb viral RNA genome tightly wrapped with N protein; the 241 kDa RNA-dependent RNA polymerase L; and a 30 kDa phosphoprotein, P. Full transcriptional activity can be reconstituted by recombining these three purified components, resulting in synthesis of a short leader (47 nt) and five viral mRNAs (Emerson, 1987).

P protein must be phosphorylated in order to become transcriptionally active. The essential phosphorylation is carried out by the ubiquitous cellular kinase casein kinase II (CKII) (Barik & Banerjee, 1992). Phosphorylation by CKII in vitro reaches a maximum value of 2 mol of phosphate/mol of P protein. Simultaneous determination of the kinetics of phosphorylation and transcriptional activation indicated that two residues were phosphorylated independently, and phosphorylation of either or both was sufficient to confer activity (Gao & Lenard, 1995a). Substitution of S60 and T62 with D residues was sufficient to confer full transcriptional activity in the absence of any phosphorylation by CKII. Indeed, the fully active S60D/T62D mutant was no longer a substrate for CKII, suggesting that these were the residues phosphorylated by CKII (Gao & Lenard, 1995a). Viral P protein contains 2−3 additional phosphates at other positions, but these were shown to be transcriptionally irrelevant (Gao & Lenard, 1995a).

Gel filtration and cross-linking studies showed that phosphorylation of P by CKII resulted in the conversion of monomeric P protein into well-defined homomultimers under transcription conditions (Gao & Lenard, 1995a). Multimer formation was essential not only for transcriptional activity but also for binding of P to the other two transcriptional components. L protein combined with a single P multimer to form a 1:1 complex that bound to the N-RNA template more strongly than did the P multimer alone (Gao & Lenard, 1995b).

The stoichiometry of the active multimer could not be determined from our previous gel filtration studies (Gao & Lenard, 1995a). In contrast, Sendai virus P protein was recently shown to be trimeric in transfected cells using an epitope dilution technique (Curran et al., 1995). In this report, we have used a similar approach, a His-tag dilution method, to show that the transcriptionally active form of VSV P protein, like the Sendai protein, is a trimer. The stability of this trimer was characterized under a variety of conditions of ionic strength and pH, and the association constants for trimerization of several partly active mutants were determined. These were found to correlate well with transcriptional activity. The CD spectra of P protein before and after CKII phosphorylation were found to be identical, consistent with the idea that stabilization of trimers is largely electrostatic, as inferred from the exchange studies.

MATERIALS AND METHODS

Purification of VSV and Viral Proteins. Growth of VSV (Indiana serotype, Mudd-Summers strain) in BHK-21F cells, purification of virions, disruption and purification of transcribing viral nucleocapsids, and purification and assay of nucleocapsid components were carried out as previously described (Gao & Lenard, 1995a).

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Purification of Recombinant P Proteins from Escherichia coli. Wild-type and mutant recombinant P proteins were expressed as His-tag fusions in *E. coli* and purified using Ni beads as described previously (Gao & Lenard, 1995a,b).

Proteolytic Removal of the His-Tag from Recombinant P Proteins. The His-tagged P proteins were digested with enterokinase (Invitrogen) at 1 unit/20 μg of protein in 50 mM Tris (pH 8.0), 1 mM $CaCl_2$, 0.1% Tween-20 at 32 °C for 16 h. The untagged P protein migrates faster than the tagged protein on 10% SDS−PAGE, providing the basis for the His-tag experiments described below.

His-tag Dilution Assay. Reactions were carried out in transcription buffer (0.1 M NaCl, 50 mM Tris, 5 mM $MgCl_2$, 4 mM DTT, pH 8.0); 1 μg of wild-type (wt) His-tagged P protein was mixed with increasing amounts of untagged wt P and then phosphorylated with CKII (Calbiochem) as described previously (Gao & Lenard, 1995a). Multimers containing tagged subunits were isolated from the reaction mixture by binding to Ni beads using the "bead method" described previously (Gao & Lenard, 1995b) and analyzed by SDS−PAGE. Densitometry was performed after staining with Coomassie blue, using a model 670 Bio-Rad densitometer. Band intensities were related to protein content by comparison with standard curves, which were determined separately for each tagged and untagged protein.

Exchange Assay. The standard reaction mixture contained 1 μg of CKII-treated (trimeric) His-tagged wt P protein and 1 μg of trimeric untagged P in 200 μL of transcription buffer. Tagged and untagged protein were mixed together at time $t = 0$ and incubated at 4 °C. At the indicated times tagged trimers were recovered from the reaction mixture using Ni beads and analyzed on SDS−PAGE as described above. Ionic strength was varied by varying NaCl content (10−500 mM). pH was varied by changing the buffer: 50 mM citrate (pH 4.0), 50 mM MES (pH 6.0), or 50 mM CAPS (pH 10.0). To assay for the effect of L protein on subunit exchange, 1 μg of a mixture of tagged and untagged multimeric P was preincubated with 3 μg of viral L protein.

Determination of Association Constants for Trimerization. To determine the association constant (K_{ass}) for the partially active mutant proteins (S60D, T62D, S60A, T62A, and S60A/T62A), 20 or 200 μg of each protein was used at a tagged:untagged ratio of 1:100. The tagged and untagged components were exchanged to equilibrium by incubation in 2 mL of transcription buffer for 30 min at 32 °C. Tagged molecules were harvested from the reaction mixture on Ni beads, and the amount of associated untagged protein was determined from SDS−PAGE as described above. The concentration of monomers and trimers could then be calculated, based on the assumption that all associated untagged molecules were present in trimers consisting of one tagged and two untagged subunits. Excess tagged molecules, i.e., those not accounted for by such trimers, were taken to constitute the monomer pool. K_{ass} was then calculated assuming the equilibrium:

$$3P \rightleftharpoons P3$$

$$K_{ass} = [P3]/[P]^3$$

For each mutant, K_{ass} was determined from the average of three measurements at 20 μg and three at 200 μg.

CD Spectroscopy of P Protein. CD spectra were measured at 25 °C on an Aviv model 620S spectropolarimeter using a 1 mm quartz cell. The measurements were performed at several protein concentrations in 10 mM KH_2PO_4 (pH 7.8), 100 mM KF, 1 mM EDTA.

Protein concentrations used to calculate molar ellipticities were determined under denaturing conditions from the absorbance at 294 nm, by making use of the known amino acid composition of the protein and the accurately determined extinction coefficients for tryptophan and tyrosine residues (Gill & von Hippel, 1989). The absorbance was determined as the difference spectrum between identical concentrations of protein in 6 M guanidine·HCl at pH 7.1 and 12.5. After correcting for base-line absorbance, A_{294} was related to concentration (C) using the relationship:

$$C(\text{mol/L}) = A_{294}/(2357\text{Y} + 830\text{W})$$

where Y and W are the number of tyrosine and tryptophan residues, respectively, per protein molecule (10 and 5, respectively, for P protein; Hudson et al., 1986).

Analysis of CD Spectra. The CD spectra were analyzed in two ways: (i) by the Selcon program of Sreerama and Woody (1993), using as reference the 16 proteins and polyglutamic acid suggested by the authors; assignments of secondary structure was done as suggested by Kabsch and Sander (1983); (ii) by the constrained method of least squares using the LINCOMB program of Perczel et al. (1992). The spectra for α-helix, β-turn, β-pleated sheet, and random coil were as suggested by Brahms and Brahms (1980). For further discussion of these techniques, see Greenfield (1996).

RESULTS

The P Protein Multimer Is a Trimer. In order to determine the stoichiometry of P multimer, a His-tag dilution method was used. This method relied upon our previous findings that the His-tag had no detectable effect on transcriptional activity (Gao & Lenard, 1995a) and that it could provide the basis for an assay to measure binding of P protein to other transcriptional components (Gao & Lenard, 1995b).

Unphosphorylated (i.e., monomeric) wt P was treated with enterokinase to remove its His-tag. Untagged P protein separated from the His-tagged protein as a faster migrating band in 10% SDS−PAGE. The untagged form was added in increasing amounts to a constant amount of the tagged form, and the mixtures were then activated by phosphorylation with CKII, resulting in complete multimer formation (Gao & Lenard, 1995a), which was confirmed by gel filtration (data not shown). Multimers possessing a tagged subunit were recovered on Ni beads, subjected to SDS−PAGE, stained with Coomassie blue, and quantitated by densitometry. The ratio of untagged:tagged monomers in the retained multimers rapidly reached a limit of 2:1, indicating formation of a trimer (Figure 1).

Exchange of Subunits between P Trimers. The rate of exchange of monomers between assembled P protein trimers was determined as a relative measure of trimer stability. Exchange was measured between preformed tagged and untagged trimers. In order to measure exchange, aliquots of His-tagged and untagged wt P protein were trimerized separately by CKII. The assembled trimers were then mixed together, and the tagged molecules were recovered on Ni beads after various periods of incubation. Exchange between tagged and untagged trimers was indicated by the presence of untagged P in the bound fraction. As shown in Figure 2A, exchange of subunits occurred fairly rapidly even at 4

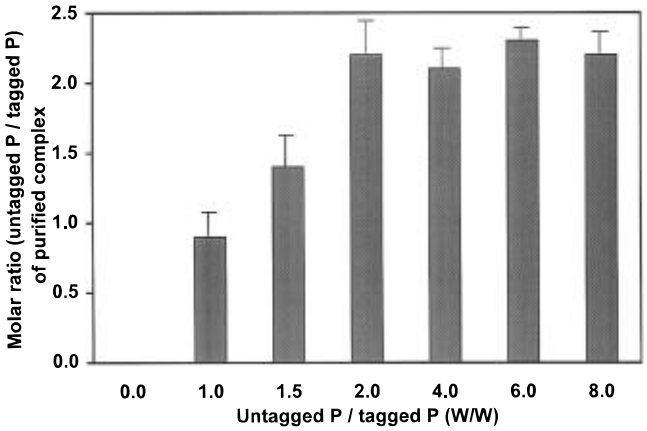

FIGURE 1: His-tag dilution experiment showing that P protein forms a trimer. Untagged wt P protein was added to His-tagged P protein in the ratios indicated. Mixtures were phosphorylated with CKII, and tagged P was recovered from the reaction mixture using Ni beads. Bead-associated tagged and untagged P were separated on SDS−PAGE, stained with Coomassie blue, and quantitated by densitometry. Data show the average and standard deviations from three experiments. See Materials and Methods for details.

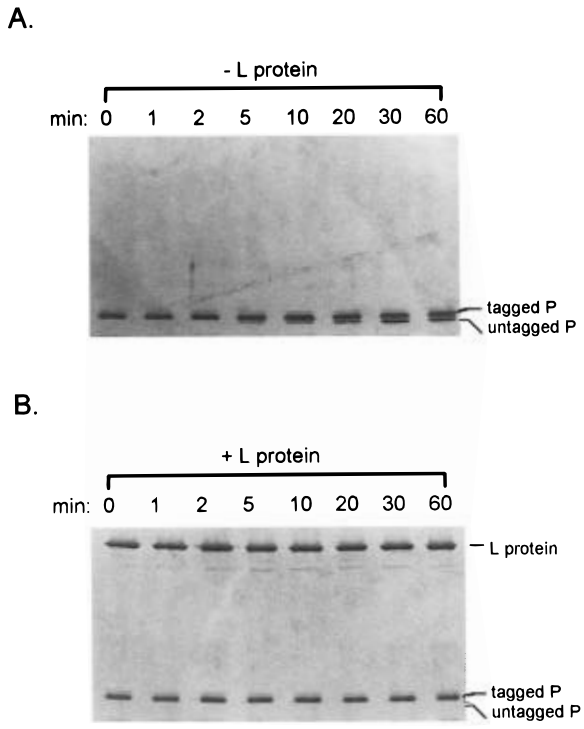

FIGURE 2: Subunit exchange experiment. Tagged and untagged trimeric (CKII-treated) wt P protein were added together in equal amounts at $t = 0$. Tagged P protein was recovered from the reaction mixture at indicated times, and associated untagged P was quantitated after separation on SDS−PAGE. (A) Exchange in transcription buffer at 4 °C. (B) Same, after preincubation with L protein. See Materials and Methods for details. At equilibrium, the bead-associated ratio of tagged:untagged P molecules is predicted to be 0.75.

°C, nearing completion by 30 min. Exchange at 32 °C was too rapid to determine conveniently (not shown).

In previous work we showed that the P trimers formed 1:1 complexes with L protein. These complexes were the only form of L that could bind to the N-RNA template, suggesting that they are functional in transcription. Further, the P trimer:L complexes bound to the template more strongly than did P trimers alone (Gao & Lenard, 1995b). In order to test the effect of L protein on P trimer exchange, a saturating amount of L protein was preincubated with

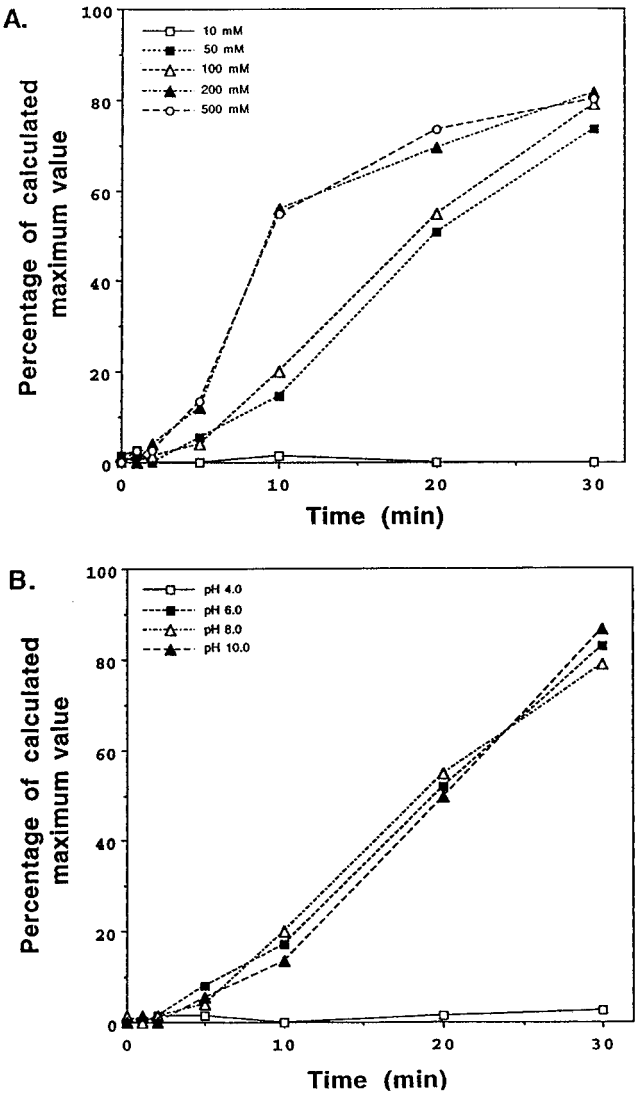

FIGURE 3: Kinetics of subunit exchange at different salt concentrations (A) or pH values (B). See Materials and Methods for details.

tagged and untagged activated wt P trimers, which were then mixed and tested for subunit exchange as above. Results are shown in Figure 2B. The presence of L stabilized the P trimers markedly; no subunit exchange could be detected during 60 min of incubation at 4 °C.

Figure 3 shows the effects of varying ionic strength and pH on the rate of trimer exchange. Although gel filtration showed that P protein remained trimeric at each salt concentration tested (data not shown), no exchange was detected at 10 mM NaCl (Figure 3A), indicating a stabilized trimer. Exchange at 50 and 100 mM NaCl showed similar kinetics, with a half-time of ca. 20 and 17 min, respectively. The exchange rate was considerably faster at 200 and 500 mM NaCl, with a half-time of ca. 8 min. These results indicate that electrostatic effects play a major role in trimer stabilization.

No differences in exchange rates were observed when the exchange assay was performed at pH 6.0, 8.0, or 10.0 (Figure 3B). However, no untagged P was detected in the bound fraction at pH 4.0. This result could arise either by stabilization of the trimer, as was observed in the presence of L protein (Figure 2B) or at low ionic strength (Figure 3A), or by complete dissociation of the trimers into monomers. Gel filtration showed that P protein eluted as a monomer at pH 4.0 (not shown). The trimer is thus stable

at pH 6 but dissociates at pH 4, suggesting that one or more carboxylate groups are involved in the electrostatic stabilizing interactions.

It was previously reported that the double mutant S60D/T62D was fully transcriptionally active without phosphorylation by CKII; in fact, it was no longer a substrate for CKII phosphorylation (Gao & Lenard, 1995a). It was therefore of interest to perform similar exchange experiments using this "pseudo-wild-type" mutant. Again, exchange was measured by the time-dependent appearance of untagged mutant protein in association with the tagged form on Ni beads. Similar curves to those in Figure 3 were obtained, showing similar dependence on ionic strength and pH (data not shown). The exchange rates were consistently about 20% slower, however, under all conditions where exchange was measurable. Thus, the half-time for exchange of S60D/T62D in transcription buffer was ca. 21 min, compared to 17 min for CKII-activated wild-type protein.

It may be noted that all the exchange kinetic curves shown in Figure 3 are sigmoidal. This is difficult to interpret in terms of symmetric trimers, consisting of three identical subunits. It seems likely that the sigmoidal shape is an artifact arising from the His-tag. The first detectable exchange must be initiated by the dissociation of a tagged subunit from an all-tagged trimer. Dissociation of the second tagged subunit, on the other hand, involves dissociation from a trimer possessing two tagged and one untagged subunits. The sigmoidal kinetics shown in Figure 3 suggest that this occurs more readily than dissociation from the all-tagged trimers, perhaps due to association between the His-tags. While this interaction evidently has no effect on biological activity or binding ability to other transcriptional components (Gao & Lenard, 1995a,b), it does preclude any meaningful analysis of exchange kinetics. Exchange of the third subunit cannot be measured in this assay, since it produces a trimer devoid of His-tags. The exchange rates between untagged trimers are presumably somewhat faster than those shown in Figure 3.

Alanine (A)- and Aspartate (D)-Substituted Mutants Are Partially Trimeric without Phosphorylation. By substituting A residues for phosphorylatable target S residues, a protein can be prepared that is often considered to represent the unphosphorylated form. We were puzzled by the observation that the mutants S60A, T62A, and S60A/T62A all possessed low but significant amounts of transcriptional activity compared to the unphosphorylated wt form. Activity levels of 5−12% of wt were found when these mutant proteins were present in the transcription assay in standard amounts (Table 1; Gao & Lenard, 1995b). Further, these mutants exhibited up to 29% of wt activity in the in vitro assay if a 10-fold excess of the mutant protein was present (Table 1). Minireplicon assays of the S60A/T62A mutant in cells, in which the protein was overexpressed and lacked the His-tag, showed replicational activity levels around 50% of wt (G. Wertz, J. Lenard, and Y. Gao, unpublished observations), consistent with the results from the in vitro assay.

If trimers are the only transcriptionally active species of P protein, then the weakly active A mutants must be partly trimeric under conditions of the transcriptional assay, without any modification by CKII. Indeed, none of the A mutants are substrates for CKII in our hands (Gao & Lenard, 1995b). The ability of the A mutants to form trimers was tested with a His-tag dilution assay, modified to detect small amounts of trimer: A tagged/untagged ratio of 1:100 was used to enhance sensitivity, and the mixture was incubated at 32 °C for 30 min to ensure complete equilibration of exchanging subunits between tagged and untagged trimers. The proportion of total protein that was trimeric was then determined from the gels, and the K_{ass} value for each mutant was calculated as described in Materials and Methods. Results are shown in Table 1. The values for K_{ass} determined for each mutant correlated well with their transcriptional activities. This constitutes strong evidence that the trimer is the only transcriptionally active form of P protein. Thus, all the A mutants differ importantly from the unphosphorylated wt protein in possessing a measurable tendency to trimerize.

Table 1: Transcriptional Activities and K_{ass} for Trimer Formation of P Protein Mutants

		relative transcription activity	
	K_{ass}, mM^{-2}	1×	10×
wild-type, unphosphorylated	0[c]	0	
phosphorylated	∞[d]	100	
S60D	8.8 ± 3.0	40[a]	98[a]
T62D	7.9 ± 1.3	42[a]	101[a]
S60A	0.37 ± 0.10	9[b]	27
T62A	0.45 ± 0.07	5[b]	19
S60A/T62A	1.12 ± 0.28	12[b]	29

[a] From Gao and Lenard (1995a). [b] From Gao and Lenard (1995b). [c] Trimer undetectable. [d] Monomer undetectable.

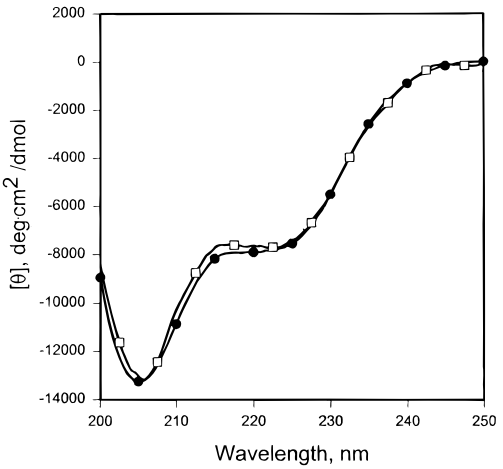

FIGURE 4: CD spectra of wt P protein before (●) and after (○) phosphorylation by CKII. See Materials and Methods for details.

Phosphorylation Does Not Alter the CD Spectrum of P Protein. The CD spectrum of wt P protein was determined before and after complete phosphorylation by CKII. Results are shown in Figure 4. No difference was detected between the spectrum from monomeric unphosphorylated P protein and that from the phosphorylated, trimeric, transcriptionally active form. CD spectra were also determined for the transcriptionally active, trimeric mutant S60D/T62D and for the predominantly monomeric mutant S60A/T62A. CD spectra for these mutants closely resembled those for the phosphorylated and unphosphorylated wt proteins (Table 2).

The CD spectra obtained in this study were analyzed by two different methods for predicting secondary structure (Table 2). The two methods agree fairly well in predicting a helical content of ca. 30% in all the P proteins studied. Predictions of the other structural motifs were quite divergent, however (Table 2). This is not surprising, since most methods, including the ones used here, predict helical content more successfully than they predict other motifs (Greenfield, 1996).

Table 2: Analysis of CD Spectra of P Protein[a]

	percent predicted secondary structure							
	α-helix		β-sheet		β-turn		random	
protein	A	B	A	B	A	B	A	B
wt, unphosphorylated	26	31	21	15	30	6	25	48
phosphorylated	28	32	20	13	28	7	24	48
S60A/T62A	32	37	20	6	27	8	24	49
S60D/T62D	29	34	19	11	39	7	25	48

[a] A = Selcon method (see Materials and Methods). [b] B = Constrained method of least squares (see Materials and Methods).

It may be noted that analyses of the P protein sequence by the Chou−Fasman and Garnier−Osguthorpe−Robson methods (Fasman, 1989) yield values of 41% and 37% helix, respectively. The two methods agree in their predictions of helix over only 20% of the sequence, however: residues 23−33, 38−47, 80−85, 113−118, 154−162, and 242−251.

DISCUSSION

The experiments reported in this paper provide further characterization of the effects of phosphorylation by CKII on P protein. This phosphorylation promotes the assembly of P protein trimers (Gao & Lenard, 1995a). The trimers are the transcriptionally active species, as shown in this paper by the correlation between the association constants for trimerization and the transcriptional activity of several weakly active point mutations (Table 1). Previous work showed that only the trimeric form of P protein could form a complex with L protein or bind to the N-RNA template (Gao & Lenard, 1995b).

The intriguing question arises, of how the introduction of one or two phosphates into an extremely negative amino acid sequence (the 10 residues 58−67 already contain six negative side chains before phosphorylation) can promote this kind of specific self-association. The CD spectra of monomers and trimers were indistinguishable (Figure 4, Table 2), indicating that extensive conformational rearrangements do not occur. The exchange measurements suggest that the stabilizing interactions between the trimer subunits are largely electrostatic and involve carboxylate groups (Figure 3). Involvement of arginine residues may also be inferred since trimer stability was unchanged in the range pH 6−10 (Figure 3B), a range within which the other cationic residues—lysine and histidine—become uncharged. The trimer is substantially stabilized by association with L protein to form the transcriptionally competent L:P complex (Figure 2B).

It seems unlikely that the CKII-modified region itself constitutes the trimerization domain. An analysis by Curran et al. (1995) predicts that the most likely trimerization domain in VSV P protein comprises residues 1−30, which is weakly predicted to form helical coiled-coils. The CKII target region, residues 60−64, is not predicted to be helical by either the Chou−Fasman or Garnier−Osborn−Robson methods. In fact this region is likely to be disordered, based on its very negative character. A very similar CKII target region in the stem of calmodulin was shown to be disordered by nuclear magnetic resonance measurements (Barbato et al., 1992).

A more plausible idea is that trimerization is inhibited by intramolecular interactions within the unphosphorylated subunit. The CKII target sequence might itself be an inhibitory region, which interacts with the trimerization domain in the unphosphorylated state to prevent trimer formation. Phosphorylation might then be imagined to inactivate this inhibitory domain. Alternatively, the disordered CKII target region might function as a flexible "hinge" in the unphosphorylated form, permitting interactions between the N-terminal trimerization domain and an inhibitory domain on the C-terminal side of the CKII target sequence. Phosphorylation might stiffen the hinge, disrupting the interaction. Experiments to test these ideas are in progress.

It is noteworthy that no differences in secondary structure could be detected between the CD spectra of monomeric and trimeric P protein (Figure 4). This finding contrasts sharply with a recent report on the P protein of VSV_{NJ} (Das et al., 1995), which claimed that unphosphorylated P protein was 27% helical, while phosphorylated P was 48% helical—a quite unprecedented change of 56 residues into a helical conformation as a consequence of the introduction of one or two phosphate groups. However, in further work this group has found no such differences between the phosphorylated and unphosphorylated forms of the P protein of VSV_{Ind} (A. K. Banerjee, personal communication). This raises the possibility that the P proteins of these two VSV serotypes, which possess remarkably little sequence homology, may respond to phosphorylation in fundamentally different ways. It may be noted that the lack of any detectable change in secondary structure upon phosphorylation, as reported here, fits well with the other findings reported in this paper, namely, stabilization of the trimers by electrostatic interactions, which generally occur at surface sites and do not require extensive conformational rearrangements.

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