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Phosphorylation-Dependent Mobility Shift of Proteins on SDS-PAGE is Due to Decreased Binding of SDS

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While many eukaryotic and some prokaryotic proteins show a phosphorylation-dependent mobility shift (PDMS) on SDS-PAGE, the molecular mechanism for this phenomenon had not been elucidated. We have recently shown that the distribution of negatively charged amino acids around the phosphorylation site is important for the PDMS of some proteins. Here, we show that replacement of the phosphorylation site with a negatively charged amino acid results in a similar degree of the mobility shift of a protein as phosphorylation, indicating that the PDMS is due to the introduction of a negative charge by phosphorylation. Compared with a protein showing no shift, one showing a retarded mobility on SDS-PAGE had a decreased capacity for SDS binding. The elucidation of the consensus sequence ($\Theta X_{1-3}\Theta X_{1-3}\Theta$, where Θ corresponds to an acidic function) for a PDMS suggests a general strategy for mutagenizing a phosphorylatable protein resulting in a PDMS.

Key Words : Electrophoretic mobility shift, Protein phosphorylation, SDS-PAGE, Signal transduction

Introduction

Phosphorylation is the most important protein modification in signal transduction pathways in all organisms. In bacteria, one of such signal transduction systems is the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) whose phosphorelay proceeds sequentially from PEP to Enzyme I, HPr, EIIA, EIIB, and finally to the incoming sugar that is transported through EIIC across the membrane and concomitantly phosphorylated. In addition to its primary functions in sugar uptake and phosphorylation, this complex system carries out numerous regulatory functions through protein-protein interactions.[1-8] These regulatory interactions involving proteins of the PTS have been shown to be dependent on the phosphorylation state of the PTS components.

Analysis of the *E. coli* genome has revealed a parallel PTS that has been referred to as the nitrogen PTS; it consists of EI^{Ntr} encoded by *ptsP*, NPr encoded by *ptsO*, and $EIIA^{Ntr}$ encoded by *ptsN* which are homologues of the carbohydrate PTS components EI, HPr, and EIIA, respectively.[9-11] Several regulatory roles of the nitrogen PTS were revealed in some bacteria.[12-17] The state of phosphorylation of proteins in this system is also crucial to the regulation phenomena.

The mobility of most eukaryotic proteins is shifted on SDS-PAGE when they become phosphorylated; we refer to this as a phosphorylation-dependent mobility shift (PDMS). This PDMS is also observed with some proteins, including PTS proteins, in bacteria.[18-20] Importantly, the explanation for this phenomenon has never been explored. In the present work, we elucidate the precise mechanism of the PDMS. We find that it involves an interplay between the negative charge of a phosphate group with vicinal negatively charged amino acids, resulting in an alteration in the number of SDS molecules bound to the protein under SDS-PAGE conditions. These findings provide a molecular mechanism for the PDMS.

Experimental Section

Bacterial Strains, Plasmids, and Culture Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultured as described previously.[12]

Purification of Overexpressed Proteins. Purification of His-tagged proteins (His-$EIIA^{Ntr}$(A132E&L131E), His-$EIIA^{Ntr}$(I35E), His-$EIIA^{Ntr}$(V13E&S21E), His-$EIIA^{Ntr}$(I94E), His-$EIIA^{Ntr}$(S12E&R22E), His-EI, His-HPr, His-HPr(K79E), His-$EIIA^{Glc}$ and His-$EIIA^{Glc}$(H90E)) was accomplished as described previously.[21] *E. coli* GI698 harboring expression vectors were grown and protein expression was induced as described previously for overproduction of other proteins.[22] His-tagged proteins were purified using BD TALON™ metal affinity resin (BD Biosciences Clontech) according to the manufacturer's instructions and bound proteins were eluted with binding buffer containing 200 mM imidazole. The fractions containing His-tagged proteins were pooled and concentrated in a 3 K Macrosep centrifugal concentrator (Pall Gelman Laboratory). To obtain homogeneous proteins (> 98% pure) and to remove imidazole, the concentrated pool was chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (GE Healthcare Life Sciences) equilibrat-

Table 1. *Escherichia coli* strains and plasmids used in this study

Strains or Plasmids	Genotype or Phenotype	Source or Reference
Strains		
GI698	F⁻λ⁻*lacI*^{q}*lacPL8 ampC*::P_{trp} cI	22
Plasmids		
pRE1	Expression vector under control of λP_L promoter, Amp^{r}	23
pPR3H	pRE1-based expression vector for EIIA^{Glc} with N-terminal 6 histidines	Lab stock
pSP100H	pRE1-based expression vector for HPr with N-terminal 6 histidines	Lab stock
pCR3H	pRE1-based expression vector for EIIA^{Ntr} with N-terminal 6 histidines	13
pPR3H(H90E)	pRE1-based expression vector for EIIA^{Glc}(H90E) with N-terminal 6 histidines	This study
pSP100H(K79E)	pRE1-based expression vector for HPr(K79E) with N-terminal 6 histidines	This study
pCR3H (A123E&L131E)	pRE1-based expression vector for EIIA^{Ntr}(A123E&L131E) with N-terminal 6 histidines	This study
pCR3H(I35E)	pRE1-based expression vector for EIIA^{Ntr}(I35E) with N-terminal 6 histidines	This study
pCR3H(I94E)	pRE1-based expression vector for EIIA^{Ntr}(I94E) with N-terminal 6 histidines	This study
pCR3H (V13E&S21E)	pRE1-based expression vector for EIIA^{Ntr}(V13E&S21E) with N-terminal 6 histidines	This study
pCR3H (S12E&R22E)	pRE1-based expression vector for EIIA^{Ntr}(S12E&R22E) with N-terminal 6 histidines	This study

ed with buffer A (20 mM Tris·HCl, pH 8.0 containing 100 mM NaCl). Fractions containing proteins were pooled and concentrated. Purified proteins were stored at −80 °C until use. Protein concentrations were determined by the Bradford assay (Bio-Rad).

Determination of the Amount of SDS Bound to Protein. For SDS determination, proteins were equilibrated with the running buffer (50 mM Tris, pH 6.8) containing 10 mM SDS at room temperature for 2 h and then chromatographed through an FPLC Superose 12 column equilibrated with the same Tris/SDS buffer. Determination of SDS was performed using a quantitative methylene blue extraction method.[24] 20 20 μL of a 1/5 diluted fraction was added to 280 μL of 50 mM Tris, pH 6.8 and mixed with 2.5 ml of 0.0024% aqueous methylene blue. Each aliquot was extracted with 10 mL chloroform and the absorbance of the organic layer at 655 nm was measured. Standard curves were prepared by chloroform extraction of SDS concentrations from 0-30 μg. The BCA assay (Pierce) was used for protein determination according to the manufacturer's direction.

Results and Discussion

Negatively Charged Amino Acids Can Mimic Phosphorylation with Respect to Phosphorylation-dependent Mobility Shift on SDS-PAGE. Phosphorylation is one of the most ubiquitous biological protein modi-fications. While the phosphorylation-dependent mobility shift of proteins on SDS-PAGE has been observed in various proteins from bacterial to eukaryotic species, the mechanism for this phenomenon had not been clarified. EIIA^{Glc} has previously been observed to change its mobility on SDS-PAGE when phosphorylated at its active site histidine,[18] whereas its paralog EIIA^{Ntr} does not exhibit such a PDMS.[25] Comparison of the amino acid sequences of the two proteins indicated that they are dissimilar in the arrangement of negatively charged amino acids surrounding the phosphory-lation site.

The region of EIIA^{Glc} in the vicinity of the active site histidine (H90) is characterized by a pair of acidic residues separated from the active site by three intervening residues (Fig. 1(a)). We tested whether the creation of the sequence $\Theta X_3 \Theta X_3 \Theta$ (Θ and X denote a negatively charged amino acid or a phosphorylated residue and any amino acid, respectively) in another protein resulted in a mobility shift. Figure 1B demonstrates that the HPr mutant K79E, in fact, shows a shift relative to the wild-type protein; a similar modification of EIIA^{Ntr} created a sequence (I69D) exhibiting a PDMS.[25]

Protein phosphorylation results in both the addition of 80 Kd of mass as well as a negative charge. To determine if the PDMS specifically required phosphorylation of EIIA^{Glc}, a

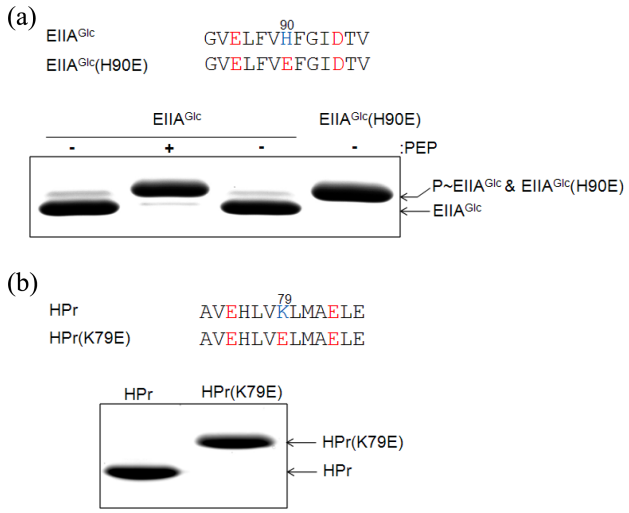

Figure 1. Substitution of a phosphorylated residue by a negatively charged amino acid can mimic the phosphor-ylation state of the protein with respect to a PDMS. (a) The mobility shift of EIIA^{Glc}(H90E) and phospho-EIIAGlc on SDS-PAGE. Purified EIIA^{Glc} and EIIA^{Glc}(H90E) were run on a 14% polyacrylamide gel under denaturing conditions. Phosphorylation of EIIA^{Glc} was carried out in the presence of purified EI, HPr and 1 mM PEP at 37 °C for 1 min. (b) The mobility shift of HPr(K79E) on SDS-PAGE. Purified HPr and HPr(K79E) were run on a 4-20% gradient polyacrylamide gel under denaturing conditions. Gels were stained with Coomassie Brilliant Blue.

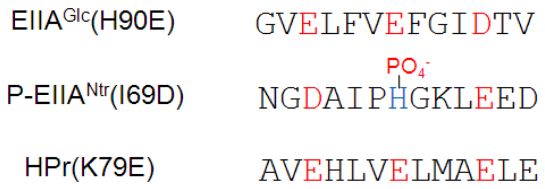

Figure 2. Amino acid sequences near negatively charged residues whose formation results in a mobility shift of proteins on SDS-PAGE.

replacement mutant (H90E), was constructed. As was the case with phosphorylated EIIA^{Glc}, the H90E mutant protein also showed a retarded mobility on SDS-PAGE without phosphorylation (Fig. 1(a)). This result suggests that the PDMS is due to the negative charge formed by phosphorylation. Similarly, the HPr point mutant protein, HPr(K79E), has symmetrically placed negatively charged amino acids with respect to Glu79 and exhibited a significant shift relative to the native protein (Fig. 1(b)).

A comparison of the sequences of several proteins that display a shift, phospho-EIIA^{Glc}, HPr(K79E) and phospho-EIIA^{Ntr}(I69D),[25] indicated that there was no sequence similarity in the intervening regions of the negatively charged amino acids (Fig. 2). Thus, charge rather than sequence appears to be the determining factor for a PDMS. Similarly, another modification resulting in a negative charge, like the addition of a sulfonic acid derivative to a residue, also led to a shift of the modified protein on SDS-PAGE.[19]

The Length of the Gap Region Between Symmetrically Placed Negatively Charged Amino Acids is an Important Factor in the Mobility Shift Phenomenon. All of the strongly shifted proteins (phospho-EIIA^{Glc}, phospho-EIIA^{Ntr} (I69D) and HPr(K79E)) had three negatively charged amino acids with three intervening residues (Fig. 2). To examine whether the number of intervening amino acids affects the mobility shift, mutant proteins with various gaps were prepared (Fig. 3A). While mutant forms of EIIA^{Ntr} with gaps 1-3 ($\Theta X_{1\text{-}3}\Theta X_{1\text{-}3}\Theta$) displayed shifts, those with a gap 0 or 4 did not, suggesting that the spacing of negatively charged amino acids is an important factor (Fig. 3(b)). Further, the length of the gap influences the extent of the shift (Fig. 3(b)). This supports the notion that the arrangement, as well as the number, of negatively charged amino acids affects the mobility shift.

A Protein Displaying a Mobility Shift Binds Less SDS. It has been shown that the number of SDS molecules bound to a protein is directly related to the mobility of the protein on SDS-PAGE.[24] Therefore, we explored the possibility that proteins exhibiting a mobility shift also bind less SDS. For this purpose, we chose to examine the pair of wild-type HPr and HPr(K79E) which differ in mobility on SDS-PAGE. SDS binds to HPr in a ratio of about 1.5 g SDS per g protein (Fig. 4), consistent with the previous finding that most proteins bind SDS in a ratio of 1.4 g SDS per g protein by hydrophobic interaction.[26] However, the ratio of SDS bound to the HPr(K79E) mutant protein significantly decreased (1.14 g/g) (Fig. 4). Therefore, this result strongly suggests

Figure 3. Effect of the length of the gap region on the mobility shift of proteins on SDS-PAGE. (a) The constru-ction of EIIA^{Ntr} mutant proteins with various lengths of the gap region between three negatively charged amino acids. (b) The mobility shift of EIIA^{Ntr} mutant proteins with various lengths of the gap region. Purified EIIA^{Ntr} and mutant proteins were run on a 4-20% gradient polyacryl-amide gel under denaturing conditions and stained with Coomassie Brilliant Blue. The migration distances of mutant forms of EIIA^{Ntr} on SDS-PAGE were measured compared to the position of unmodified EIIA^{Ntr} protein on gels. The degree of mobility shift is presented below the protein band for each mutant as shift distance (distance, in mm, from EIIA^{Ntr} to the mutant protein).

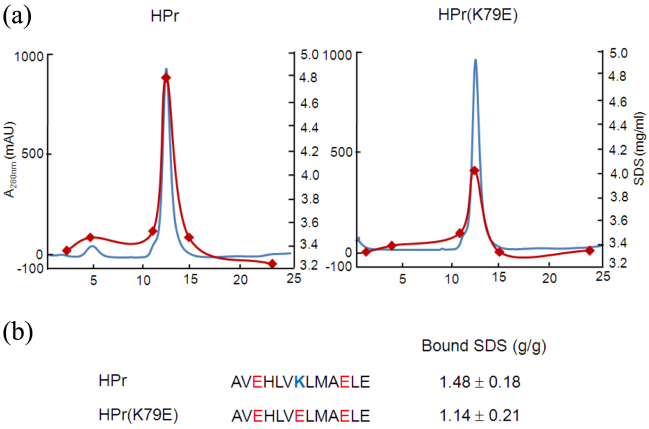

Figure 4. Measurement of SDS bound to HPr and HPr(K79E). (a) Elution profiles and SDS-binding levels of HPr and HPr(K79E). The absorbance at 280 nm (blue, arbitrary units) and the SDS concentration (red, mg/mL) were measured. Fractions of 1 mL were collected and amounts of bound SDS were determined using a methylene blue extraction method as described in "Experimental Section". (b) Estimated amounts of bound SDS. Means ± standard deviations of 3 independent experiments are shown.

that the phenomenon of a PDMS is related to a decrease in the number of SDS molecules bound to a protein containing additional negative charge adjacent to pre-existing negative charges. Since the SDS molecule has a negative charge, negative charged residues may repel SDS molecules.

There still remains a question why only proteins with 1-3 intervening residues (gaps 1-3), but not gap 0 or gap 4,

between the phosphorylation site and nearby negatively charged amino acids show the PDMS on SDS-PAGE.

Recently, it was reported that an α-helix of a protein is not completely melted even when SDS was present.[27] Therefore, one possibility is that only a protein with gaps 1-3 might prevent the typical binding of SDS molecules and thus delay the migration of the protein where the distance between the phosphorylation site and negatively charged amino acids is not too close but within one α-helical turn.

The PDMS phenomenon of eukaryotic proteins on SDS-PAGE can be explained in the same way. For example, the murine Bcl-2 family protein BAD has three phosphorylation sites (Ser122, Ser136 and Ser155) and this protein also exhibits a PDMS on SDS-PAGE.[28] While the S122A and S136A double mutant still showed the same degree of mobility shift on phosphorylation as does the wild-type protein, the S155A mutant protein did not show a PDMS. While there are no negatively charged amino acids near S122 and S136 within 5 residues on either side, negatively charged amino acids are clustered close to S155 (M**S**DEFEG, the phosphorylation site in bold face and negatively charged residues underlined). The sequence near S155 fits the consensus $\Theta X_1\Theta X_1\Theta$ on phosphorylation. It is noteworthy that the cluster of negatively charged residues containing the consensus sequence is found to follow the phosphorylatable serine. This result indicates that the consensus cluster can include residues on both or on only one side of the phosphorylation site. We have recently shown that $EIIA^{Ntr}$-(K75D), where negatively charged residues are located on only one side of the phosphorylation site His73, also showed a PDMS on SDS-PAGE.[25] This supports the idea that the phosphorylation site does not have to be located in the center of the consensus sequence $\Theta X_{1\text{-}3}\Theta X_{1\text{-}3}\Theta$ in a protein showing a PDMS on SDS-PAGE. Thus, the mechanism described herein might explain the PDMS behavior of all proteins. A strategy to mutagenize a protein to show a PDMS without affecting its activity could simplify studies of the *in vivo* phosphorylation state of a signal transduction protein under various conditions.

Conclusion

The work described here is the first report concerning the mechanism of the mobility shift on SDS-PAGE by phosphorylation, a phenomenon observed with proteins in all life forms. Our results demonstrate that the interplay between the negative charge of the phosphate group and vicinal negatively charged amino acids is the major determinant of the phosphorylation-dependent mobility shift behavior. It was also shown that an arrangement of negatively charged amino acids that results in a mobility shift is characterized by a decreased binding of SDS.

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