Affinity properties of phosvitin: Interaction of phosvitin with serine hydroxymethyl transferase

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The affinity of phosvitin with serine hydroxymethyl transferase (SHMT), an acidic multi-subunit protein, was evaluated by measurements of enzyme activity, sedimentation velocity, steady-state fluorescence, circular dichroism and kinetic thermal stability. While the presence of phosvitin had no effect on the SHMT activity, the sedimentation coefficient of SHMT increased from 8.7 S to 12.5 S suggesting the formation of a complex at a SHMT:phosvitin molar ratio of 2:1. Based on steady-state fluorescence quenching measurements an association constant of $2.4 \pm 0.2 \times 10^5 \text{M}^{-1}$ at $25^\circ$C was obtained for the interaction of phosvitin with SHMT. The temperature dependency of the association constant in the range 15-35°C suggests the involvement of ionic forces in the interaction. The thermal inactivation of SHMT followed first order kinetics. In the presence of phosvitin the rate constant decreased and half time increased. The circular dichroism measurements suggest that phosvitin interaction does not involve pyridoxal phosphate binding domain of the enzyme. Although minor changes in the secondary structure of the enzyme were observed, the environment around aromatic amino acids did not change significantly.

The principal phosphoprotein of egg yolk is phosvitin. It is one of the products of proteolytic cleavage of vitellogenin which is synthesised under estrogen stimulus in the liver of oviparous animals. Hen egg yolk phosvitin ($M_r=34$ kDa) is probably the most extensively phosphorylated protein in nature. More than 50% of its constituent amino acids are serine residues. Most of these serine residues are phosphorylated and are present in clusters of 4-16. Owing to the presence of these phosphoserine clusters, phosvitin interacts with bivalent cations and is, therefore, thought to be the store house of metals in the egg. Till date all efforts to define the three dimensional structure of this phosphorylated domain of vitellogenin have not yielded any information, and it is thought that phosvitin has largely aperiodic structure. Cytochrome c forms a soluble complex with phosvitin with a phosvitin:cytochrome c stoichiometry of 1:20 (ref. 5). Based on these studies, it has been suggested that phosvitin acts as a scaffold on to which many molecules of cytochrome c bind. Since phosvitin is highly acidic, it is not surprising to find molecules such as poly-L-lysine and protamine exhibiting affinity towards it.

We had reported earlier that a variety of proteins and polypeptides bind to phosvitin. In general, the class of proteins interacting with phosvitin also showed affinity towards heparin. Interestingly, serine hydroxymethyltransferase (SHMT) an acidic protein with $p_I$ of 4.2 also exhibits affinity to phosvitin. The SHMT, which is a homotetrameric molecule (subunit $M_r=53$ kDa), catalyses the conversion of serine to glycine and tetrahydrofolate. This reaction involves the enzyme bound cofactor pyridoxal-5'-phosphate (PLP) [10-13]. The cytosolic SHMT is the first enzyme in the pathway for the interconversion of folate coenzymes and links amino acid, carbohydrates and nucleotide metabolism in purine biosynthesis [14].

In the present study we have investigated the nature of interaction between phosvitin and SHMT.

Materials and Methods

Purification of phosvitin was carried out according to the method of Mecham and Olcott with some modifications as reported earlier. The protein was further purified using a Mono Q column HR 5/5
(FPLC Pharmacia). Phosvitin eluted as single symmetrical peak at 0.47 M concentration of NaCl in buffer. Fractions corresponding to the peak were pooled, dialysed against Milli Q water and lyophilised. The SHMT was purified from sheep liver using CM-Sepahex and Sepharose S-200 chromatography, according to the procedure of Baskaran et al.19. This procedure yields approximately 80 mg protein per batch of 1.5 kg liver. The enzyme was homogeneous as judged by native and SDS-PAGE. The enzyme had a specific activity of 5.6 μmoles of formaldehyde formed/min/mg protein.

**Assay of SHMT activity**

The procedure used to estimate the enzyme activity was that originally described by Taylor and Weissbach17 and modified by Manohar et al.18. [H4] folate was prepared by hydrogenation of folic acid using platinum oxide (50 mg) in glacial acetic acid as the catalyst. The purity of the preparation was estimated to be more than 95% by its molar extinction coefficient (ε) of 28 × 104 M−1cm−1 at 297 nm. Protein concentration was determined by the method of Lowry et al.21 with BSA as standard.

**CD spectra**

CD measurements were made using a JASCO J 20-C automated spectropolarimeter. The instrument was calibrated with d-10 camphor sulfonic acid22. Slits were programmed to yield 10 Å band width at each wavelength. The SHMT CD spectra were plotted as mean residue ellipticity [θ] MRW, using a value of 110 for MRW. The '0' value was calculated according to the method of Grenfield and Fasman23. All CD spectra were recorded in 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM DTT and using the same buffer as the blank. The spectra were recorded at 25 ± 1°C in cells of appropriate path length. The secondary structure analysis was made using the computer programme CDESTIMA provided by Prof. Fasman.

**Fluorescence quenching**

Fluorescence quenching experiments were done for the phosvitin-SHMT system to determine the association constant of the interaction. Exactly 2 ml of the enzyme (150 μg/ml) in 50 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA and 1 mM DTT was taken in a 3 ml quartz cuvette and the fluorescence emission spectrum in the range 300-400 nm was recorded by exciting at 280 nm using a Shimadzu RF-5000 Spectrofluorophotometer with a thermostated cell. The excitation and emission slit widths were 5 and 10 nm respectively. To the enzyme solution, aliquots of phosvitin (10 μl, 1.0 mg/ml) were added, and the emission spectra were recorded with constant stirring. Appropriate concentrations of phosvitin in buffer were used as controls. The fluorescence data were analyzed to obtain per cent Q (quenching) at each concentration of the ligand added. These values were also plotted as reciprocal plot. The Y intercept 1/Qmax was obtained by linear regression analysis24. The fractional saturation, b, the free ligand concentration Cf and association constant (Ks) were calculated according to Lehrer and Fasman25. The association constant was also evaluated by using the equation reported by Appu Rao and Cann26 and Appu Rao27.

**Determination of mid-point of thermal denaturation**

The thermal denaturation of SHMT in the presence of increasing concentrations of phosvitin was carried out by measuring the absorbance at 287 nm as a function of temperature using Gilford Response II Spectrophotometer from Ciba Corning, USA. A clear protein solution of 300 μg/ml of the SHMT or SHMT-PV complex was prepared in 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM 2-ME and 250 μl of this solution was used with appropriate blanks in thermal quartz cuvettes and equilibrated at 25°C. The samples were heated from 25°C to 95°C in steps of 1°C. The absorbance was monitored at 287 nm and the data averaged from three experiments. The apparent transition temperature (app Tm) was obtained from the first derivative plot using the software available with the instrument.

**Sedimentation velocity experiments**

Sedimentation velocity experiments were carried out at 59,780 rpm at 27 ± 2°C in a Spinco Model E Analytical Ultracentrifuge (Beckman, USA) equipped with phase plate, Schlieren optics and rotor temperature indicator and control unit. A 12 mm Kel F single sector center piece with quartz windows was used. Photographs were taken at regular intervals of time during the centrifugation. The plates were read using a Gaertner Microcomparator M-2000 equipped with
dual axis X-Y digital display unit. \( S_{20,w} \) apparent sedimentation coefficient was calculated according to the method of Schachman\textsuperscript{28}.

**Results**

*A The phosvitin-SHMT complex*

**Demonstration of the binding of SHMT to phosvitin**

When purified SHMT was applied to a column of phosvitin-Sepharose, previously equilibrated with 50 mM potassium phosphate buffer \( \text{pH} 7.4 \) containing 1 mM EDTA, 1 mM DTT and 50 mg/ml pyridoxal-5'-phosphate, the enzyme was bound and could be eluted from the column with buffer containing 0.2 M sodium chloride. This suggests that SHMT has affinity for phosvitin. The SHMT, present in the crude extract prepared from the sheep liver, was also found to bind to phosvitin-Sepharose matrix. However, the addition of phosvitin to SHMT in 50 mM phosphate buffer, \( \text{pH} 7.4 \), containing 1 mM EOTA and 1 mM DTT, had no effect on the catalytic activity of the enzyme.

**Sedimentation analysis of the phosvitin-SHMT complex**

The purified SHMT sedimented as a single peak with a \( S_{20,w} \) value of 8.7 S while phosvitin had a \( S_{20,w} \) value of 1.9 S. In experiments involving both proteins, the concentration of SHMT was kept at 12 mg/ml (56.3 \( \mu \)M) and that of the phosvitin was varied from 14 \( \mu \)M to 56.3 \( \mu \)M. The addition of phosvitin (14 \( \mu \)M) brought about a decrease in the area under the SHMT peak (8.7 S), while a new small peak appeared around 12.5 S, clearly indicating the formation of a complex. When the phosvitin concentration increased to give a SHMT:PV ratio of 2:1, a major peak at an \( S_{20,w} \) value of 12.9 S was observed. Further increase in the phosvitin concentration to give equimolar ratio of phosvitin to SHMT resulted in the complex peak appearing with an \( S_{20,w} \) value of 10.5. At SHMT:PV ratio of 2:1 the aggregate exhibits highest sedimentation coefficient indicating the interaction of two molecules of SHMT with one molecule of phosvitin (Fig. 1).

**Fluorescence titrations of SHMT with phosvitin**

Quantitation of the strength of the interaction was done by fluorescence titrations of SHMT with phosvitin. SHMT upon excitation at 280 nm had an emission maximum at 340 nm whereas phosvitin had an emission maximum at 355 nm. The relative quantum yield for the fluorescence emission of phosvitin was very low. The emission maximum of phosvitin is indicative of tryptophan residue in a polar environment.

The intrinsic fluorescence of SHMT was quenched upon titration with phosvitin. For the quantitation of the quenching data, the values at 320 nm were used. This was chosen to minimize the background fluorescence of phosvitin. For calculation of quenching upon the addition of phosvitin, the fluorescence intensities of phosvitin, and SHMT were measured individually and the sum of these values was subtracted from the fluorescence intensity of the complex. The percent quenching versus phosvitin concentration is shown in Fig. 1—Formation of the phosvitin-SHMT complexes (A): Sedimentation velocity profile of PV-SHMT complexes at different molar ratios from 1:1 to 1:4. (B) Plot of \( s_{20,w} \) of PV-SHMT complexes at different concentrations of PV and SHMT. [SHMT, 12 mg/ml (56.3 \( \mu \)M) was used in the cell and the concentration of phosvitin was varied such that the ratios of SHMT : phosvitin were 1:1, 1:2, 1:3 and 1:4. Photographs were taken at 7 min intervals after the centrifuge attained the maximum speed. The \( s_{20,w} \) were calculated according to the method of Schachman\textsuperscript{28}.)
The double reciprocal plot of the concentration versus per cent quenching (Fig. 2B) was constructed to measure maximum quenching. The plot of $\beta/(1-\beta)$ versus $C_T$ is shown in (Fig. 2C). The association constant obtained from the above plot was $2.43 \times 10^{-5}$ M$^{-1}$ at 25°C. The quenching data was used for the calculation of type of binding sites by plotting $\log Q/(Q_{\text{max}}-Q)$ versus $\log C_T$ (Fig. 2D). The value of the slope of the regressed line represented the type of binding sites for phosvitin on SHMT. The temperature dependence of the association constant was measured in the range 15-35°C. It was observed that at lower temperature the $K_a$ was higher. The temperature dependence of the association constant is shown in Fig. 3. The free energy change for the reaction ranged from $-7.9$ kCal/mole to $6.9$ kCal/mole. The entropy of the system was 41 e.u. in the temperature range studied. This is suggestive of the role of ionic interactions. This plot had a slope of unity which indicates single binding site on the SHMT for phosvitin.

**Effect of phosvitin on the thermal stability of SHMT**

SHMT, being a globular protein, is susceptible to denaturation by heat. Phosvitin, on the other hand is thought to possess aperiodic structure at physiological pH. The affinity properties of phosvitin are not affected by heat as indicated by the ability of heat treated phosvitin to bind proteins (Lakhey H. V., unpublished data). It was of interest to study the effect of association of phosvitin on the thermal stability of SHMT.

SHMT (150 μg) was taken in 2 ml of potassium phosphate buffer, pH 7.4; containing 1 mM EDTA and 1 mM DTT. The excitation wavelength was fixed at 280 nm. The emission spectra were recorded from 300-400 nm and fixed at 320 nm. The excitation was kept at 10 nm each and temperature of the solution was maintained at 25°C. The SHMT solution was titrated with a phosvitin solution of 2 mg/ml concentration.
SHMT. The SHMT phosvitin complex was preincubated at 50, 55, 60 and 62°C for 30 min and aliquots were taken at 5 min intervals for the assay. The percent remaining activity of SHMT was assayed. Activity of the unincubated SHMT samples was taken as 100% and the percent apparent remaining activity of heated samples (both in presence and absence of phosvitin) were calculated. Inactivation rate constants ($R_i$) were obtained from the slopes of the plots of linear regression of logarithms of relative per cent activity versus time. Phosvitin decreased the rate constant of thermal inactivation of SHMT and increased the half life. The temperature dependence of the rate constant is shown in the Arrhenius plot (Fig. 4). The inactivation of SHMT with temperature followed first order kinetics. This plot yielded the enthalpy of denatuation which was 58 Kcal for the native enzyme compared to 67 Kcal for the complex. The higher enthalpy of denaturation of the complex indicated that the association, indeed, protected the enzyme against heat denaturation.

**Effect of phosvitin on the transition temperature of SHMT**

To determine the extent of protection afforded by the interaction, the apparent $T_m$ values (mid point of the thermal inactivation profile) for the complexes for various phosvitin-SHMT ratios were determined. With the increase in temperature, there was an increase in the absorbance at 287 nm. The mid point of this transition obtained from the first derivative plots was 52°C for SHMT. Phosvitin does not exhibit any transition in this temperature range. The results of the experiment are shown in Fig. 5. With the addition of phosvitin to SHMT the app $T_m$ of the complex shifted to higher temperatures, suggesting the enhanced thermal stability of the enzyme in the presence of phosvitin.

**Effect of interaction of phosvitin on the structure of SHMT**

Although interaction of phosvitin did not have any effect on the activity of SHMT, the enzyme was observed to be more stable to heat denaturation in the presence of phosvitin. It was, therefore, of interest to analyze the changes in conformation of the SHMT due to the interaction with phosvitin. The conformational changes were monitored by measuring the CD spectra of SHMT in the visible, near UV and far UV regions in the presence of phosvitin.
In the visible region, the SHMT exhibited a positive CD band at 425 nm. This band is due to the PLP bound to the enzyme in an asymmetric environment. With the addition of phosvitin, there was no significant change in the CD spectra in this region suggesting that the interaction of phosvitin with SHMT did not affect the environment around the bound PLP (Fig. 6A).

Near UV CD spectra

The near UV CD spectrum of SHMT in the presence and absence of phosvitin is shown in Fig. 6B. SHMT exhibited negative CD bands around 262, 269 and 282 nm. With the addition of phosvitin, there were only subtle changes observed in these bands suggesting that there are no gross changes in the tertiary structure of SHMT due to its interaction with phosvitin. As seen from the fluorescence measurements, there could be a very small change in the environment of tryptophan residues of the enzyme.

The far UV CD spectrum of SHMT in the presence and absence of phosvitin is shown in Fig. 6C. The enzyme exhibits minima around 222 and 208 nm typical of a protein rich in alpha-helix. Comparison of the secondary structure, as analysed by the computer programme, is given in Table 1. With the addition of phosvitin there was a small decrease in the ellipticity values in the region 200-225 nm.

Discussion

We have shown earlier that a number of proteins from a variety of tissue extracts exhibit affinity to phosvitin. For some of these peptides/proteins where sequences are known, it was possible to locate short stretches of basic domains that are thought to participate in the interaction. It is presumed that these basic

| Table 1—Secondary structure predictions for SHMT in the presence of phosvitin |
|-----------------|---------|---------|---------|-------|
|                 | α Helix (%) | β Sheet (%) | β Turn (%) | Random coil (%) |
| SHMT           | 34.5     | 19.5    | 17.5    | 28.5   |
| SHMT+PV        | 31.0     | 20.0    | 18.0    | 31.0   |

Fig. 5—Apparent $T_m$ of SHMT as a function of the increasing concentration of phosvitin in the phosvitin-SHMT complexes.

Fig. 6—(A): Visible CD band of SHMT in the presence, (-----) and absence, (----) of phosvitin
(B): Near UV CD of SHMT in the presence, (-----) and absence, (----) of phosvitin
(C): Far UV CD of SHMT in the presence, (-----) and absence, (----) of phosvitin
domains form complementary structures to the acidic phosphorylated serine clusters present in phosvitin.

In our efforts to extend these observations to other proteins, it was noted that serine hydroxymethyl transferase (SHMT) binds to phosvitin. But it is known that phosphoserine is an active-site inhibitor of SHMT. Since phosphoserine clusters are present in phosvitin it was of interest to check whether the activity of the enzyme is inhibited by this protein. However, addition of phosvitin either in the assay or in the preincubation had no effect on SHMT activity. These results suggest the participation of surface domains rather than the active-site of the enzyme in the interaction. However, as both SHMT and phosvitin have overall negative surface charge, the interaction between these two molecules needed to be unequivocally established even though similar surface charge need not preclude them from interaction at specific locations. The binding of SHMT to phosvitin-sepharose and its elution by salt from the matrix itself provided some evidence of interaction. A more convincing demonstration of complex formation came from ultracentrifugal studies. From these experiments as well as from the thermal denaturation studies, the stoichiometry of interaction between SHMT and phosvitin was determined to be 2:1.

Further evidence for the interaction was obtained by fluorescence measurements. In these experiments, it was found that the fluorescence emission spectrum of SHMT was quenched by the addition of phosvitin. Analysis of this data indicated the existence of a single binding site on SHMT for phosvitin. The association constant derived for the interaction SHMT and phosvitin was $2.4 \times 10^4 \, \text{M}^{-1}$ at 25°C corresponding to a free energy of $-6.9 \, \text{kCal/mole}$. The presence of a single binding site on SHMT for phosvitin is surprising because the former is a homotetrameric molecule. It is possible that the domains involved in the interaction are spatially located very closely to each other in the tetrameric molecule and interact as a single unit. Such a situation can also arise, if upon interaction with phosvitin there is a strong structural perturbation that masks the sites in other subunits. Although the fluorescence data and the CD studies indicate minor structural changes in this interaction, further confirmatory studies are needed.

To sum up, it may be mentioned that phosvitin which possesses clusters of phosphorylated serine residues appear to interact with a short stretch of complementary domains present in other proteins. Although this is not a very high affinity interaction, it seems to be rather specific. This property may be useful in the biological milieu of developing embryo, where it can serve as a scaffold for anchoring important proteins. It is worth mentioning here that preformed bFGF is present in Xenopus embryos and is known to trigger mesoderm induction. Use of heparin will prevent the process of mesoderm induction by bFGF implying that a heparin-like molecule may be holding the bFGF or other molecules from action until they are required for functional purpose. Since we have shown a strong similarity between phosvitin and heparin in their ability to interact with a certain class of proteins, the above stated role may be played by this phosphoprotein in situ.

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References

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