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금속 친화성 액 이상분계 시스템에서 Phsphoprotein 분배 및 분배예측

정 봉 현

KIST 유전공학연구소

Phosphoprotein Partitioning in Metal-Affinity Aqueous Two-Phase Systems and Prediction of Partitioning Behavior

Bong Hyun Chung
Genetic Engineering Research Institute, KIST, P. O. Box 115, Yusong,
Taejon 305-600, Korea

ABSTRACT

A mathematical model has been derived and used to describe phosphoprotein partitioning in Fe(III) IDA-PEG/dextran two-phase systems. This model includes the inhibitory effects of hydrogen and hydroxyl ion concentrations on protein partitioning. For aqueous two-phase partitioning experiments, the A1 and A2 subcomponents of ovalbumin carrying two and one surface phosphoryl group(s) were purified using an immobilized metal ion affinity chromatography (IMAC). The ratio of partition coefficients in the presence and absence of Fe(III)IDA-PEG, K/Ko, increased in the pH range of 3.0 to 5.0 due to deprotonation of the second oxygen of the phosphoryl group, and above pH 5.0 declined steeply by the inhibitory binding of hydroxyl ions to the metal ion. This partitioning behavior was well-described by the mathematical model. The binding constants for formation of the complex between the phosphoryl group and the Fe(III)IDA-PEG were found to be 6.1×10^{3} M^{-1} and 2.3×10^{4} M^{-1} in the top and bottom phases, respectively. These values are 3-5 times those for interaction of Cu(II)IDA-PEG with a single surface-accessible histidine.

INTRODUCTION

Recently a metal affinity partitioning technique has been developed to selectively enhance the partitioning of metal-binding proteins in aqueous two-phase systems. The principle is based on the interactions between particular amino acids on the protein surface and metal ions attached to a metal chelating derivative of polyethylene glycol (PEG). Several attempts have been made with PEG-iminodiacetic acid bearing Cu(II)(1,2). Addition of small amount of this metallated PEG derivative to a PEG/dextran two-phase system significantly increased the partition coefficients of proteins which contain surface-accessible histidines. The detailed interacting mechanism between Cu(II)IDA-PEG and surface histidines on proteins in aqueous two-phase systems has been elucidated experimentally and theoretically by Suh and Arnold(2).

Protein-phosphorylating systems have been known to play important roles in the regulation of various cellular functions such as transmembrane nutrient and salt transport, intermediary metabolism, protein synthesis, cellular differentiation, and cytoskeletal protein activities and cell movement in eukaryotes and prokaryotes (3). Phosphorylation can affect the complex nervous system through its role in regulation of gene transcription and is also an important marker for certain disease states. Development of novel separation techniques for phosphoproteins has been in particular demand for fundamental researches to understand protein-phosphorylation system in a variety of physiological cellular processes. Andersson and Porath(4) have demonstrated that ferric ion (Fe(III)) adsorbed to iminodiacetic acid substituted agarose can be used for a selective separation of phosphoproteins due to its specific binding property for phosphoryl groups on the protein surface. More recently, Chung and Arnold(5) have developed a phosphoprotein separation technique using metal-affinity partitioning in a Fe(III)IDA-PEG/dextran two-phase system.

In this study a mathematical model that includes the inhibitory effects of hydrogen and hydroxyl ion concentrations on phosphoprotein partitioning has been developed to describe partitioning behavior and to determine the binding constants for the Fe(III)IDA-PEG/phosphoprotein complex. The ability of this model to describe the partitioning behavior has been tested with the A1 and A2 subcomponents of ovalbumin which contain two and one phosphate(s) on the protein surface, respectively.

MATERIALS AND METHODS

Materials

Ovalbumin (chicken egg albumin, grade V) was obtained from Sigma (St. Louis, USA) and used without further purification. PEG 10,000 monomethyl ether was the generous gift of Dow. Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden). All other salts and chemicals used in this study were of analytical grade. MPEG 10,000-IDA was synthesized as described previously for the syntheses of $Cu(II)_2$PEG 20,000-$(IDA)_2$ (6).

Immobilized Metal Ion Affinity Chromatography (IMAC)

A1, A2 and A3 fractions of ovalbumin were purified by IMAC using Fe(III)-IDA-Sepharose (Pharmacia LKB). The column (0.8cm×16cm) was saturated with 20mM $FeCl_3$ solution. For removal of unbound metal ions the column was washed with distilled water, 0.05M MOPS/1 M NaCl buffer (pH 7.5) and finally 0.05 M MES/1M NaCl buffer (pH 5.5). The 10 mg protein was dissolved in 10 ml 0.05 M MES/1M NaCl buffer solution (pH 5.5) and applied to the column. For elution of proteins the column was washed with 0.05 M MES/1 M NaCl buffer (pH 5.5) and continuous pH gradient formed by the gradual mixing of 0.05 M MOPS/1 M NaCl buffer (pH 7.5) with 0.05 M MES/1 M NaCl buffer (pH 5.5). After each experiment the column was regenerated with 0.1M EDTA , 1M monobasic sodium phosphate, and washed with distilled water. Protein concentrations were monitored by the determination of absorbance at 280 nm, and the eluant pH was measured using a pH meter.

A1, A2 and A3 fractions collected were dialyzed extensively against distilled water and then concentrated using an ultrafiltration kit (Amicon Co. USA) for aqueous two-phase partitioning experiments.

Partitioning Experiments

Two-phase systems were prepared from aqueous stock solutions of polyethylene glycol (40%, w/w) and dextran (13.54%, w/w). Metal-containing PEG stock solutions were prepared immediately before each experiment by adding equal molar quantities of $FeCl_3$ and PEG 10,000-IDA to PEG stock solutions. The metal-containing PEG stock solution consists of 4% Fe(III)IDA-PEG 10,000, unless otherwise specified. Aqueous two-

phase systems (2.0g total wt.) were made by combining 0.35g PEG solution, 0.65g dextran solution, and 1.00g of a solution of 0.5mg/g protein in 0.05M acetic acid-sodium acetate buffer and 0.5M NaCl. The resulting polymer compositions were 7.0% PEG and 4.4% dextran. The mixture was shaken for 30min and centrifuged for 15min to separate the phase. Samples of same volume were withdrawn from each phase, mixed with known volume distilled water and analyzed.

Protein concentrations were determined by Bradford method(7), unless otherwise stated. The corresponding phase from a two-phase system containing no protein was similarly prepared and used as a reference. Ferric ion concentration was determined colorimetrically at 510 nm(8).

RESULTS AND DISCUSSION

Fractionation of Ovalbumin Using IMAC

Native ovalbumin is composed of three protein subcomponents, A1, A2 and A3(9). The A1 component containing two phosphoserines accounts for 85% of the total protein, and A2 with one phosphoserine, 15%. Besides these two components, there are minute amounts of A3 without phosphorus. The average phosphorus content evaluated from these compositions is 1.85 mol of phosphorus per mole of protein.

Andersson and Porath(4) have fractionated native ovalbumin (Sigma, grade II) using IMAC and obtained similar composition to the above. We have used grade V ovalbumin (Sigma) which is more purified than grade II, and interestingly obtained different composition from that of Andersson and Porath. The elution profile of ovalbumin subcomponents is shown in Fig. 1. As expected, the A3 subcomponent without phosphate did not display any affinity for immobilized ferric ion. The A1 and A2 subcomponents carrying two and one phosphoryl group(s), respectively, were retarded in the IMAC column, indicating the interactions with immobilized ferric ions. In the elution profile, the A1 component represents 42% of the total protein, A2 58%, and minute amount

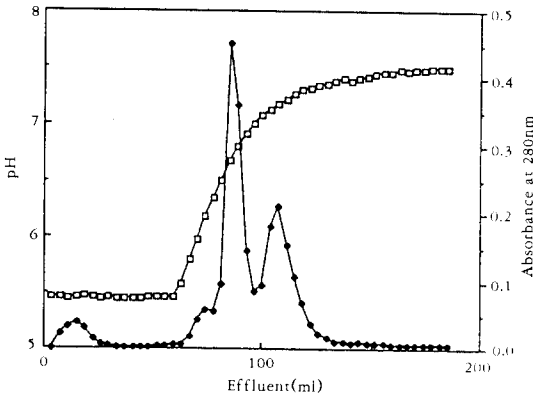

Fig. 1. Elution of ovalbumin on Fe(III)IDA-Sepharose gel using a pH gradient:(●) absorbance at 280nm:(□)pH. Chromatography: 10mg protein applied to Fe(III)-loaded IDA-Sepharose column and eluted with pH gradient formed by the gradual mixing of 0.05M MOPS/1M NaCl buffer (pH7.5) with 0.05M MES/1M NaCl buffer (pH5.5).

of A3. The purity of ovalbumin used in this study is approximately 99% according to Sigma, while grade II ovalbumin is crude. It is believed that the composition was altered in the purification process to obtain proteins of high purity. Fractions of A1, A2 and A3 which were eluted at pH 7.2, 6.6 and 5.5, respectively, were pooled, dialyzed, and concentrated for aqueous two-phase partitioning experiments. Considerable amounts of A1 and A2 components were obtained, but the A3 concentration was too low to be used for further experiments.

Partitioning of Ferric Ion in IDA-PEG(or PEG)/Dextran Two-Phase Systems

Fig. 2 shows the effect of pH on the partitioning of ferric ion in PEG/dextran two-phase systems where 4% of the MPEG was replaced by an equal molar quantity of Fe(III)IDA-PEG. The partitioning of ferric ion was strongly dependent on pH from pH 3.0 to 4.4, and reached a plateau value of approximately 21.6. To investigate the

possible interactions between PEG or dextran and free ferric ion, $FeCl_3$, at a concentration of 2.76×10^{-4} M was directly added to PEG/dextran aqueous two-phase systems containing no IDA-PEG. It is of interest that ferric ions were hardly detected in the top phase, indicating the binding of free ferric ion to dextran. The partition coefficients of free ferric ions in the pH range of 3.0 to 5.8 were less than 0.01 (data not shown). The binding mechanism between ferric ions and dextran is not yet clear but this is considered to be caused by the interactions of ferric ions with hydroxyl groups of dextran.

One of three pK values of iminodiacetic acid is 2.62 at 25℃ and 0.1 ionic strength(10). Therefore, competitive binding of hydroxyl ion against tridendate complex, Fe(III)IDA-PEG, can occur at the lowest pH 3.0, which can consequently result in a weaker binding between ferric ion and IDA-PEG. On the contrary, dextran containing hydroxyl groups would force ferric ions towards the bottom phase, resulting in a decrease in the partition coefficient of ferric ion. In case of $CuSO_4$ partitioning, copper ion was evenly partitioned in the top and bottom phases in contrast to ferric ion partitioning. Possible interactions of some metal ions including ferric ion with dextran or PEG could be elucidated through further detailed studies.

Partitioning of A1 and A2 Subcomponents in PEG/Dextran Two-Phase Systems

The aqueous two-phase extraction experiments were carried out with the purified A1 and A2 subcomponents of ovalbumin to investigate the effect of one more (or less) phosphoryl group on the protein surface on the phosphoprotein partitioning in PEG/dextran two-phase systems containing no metallated PEG derivative. As shown in Fig. 3, the partition coefficients of A1 are almost the same with those of A2 at varying pH's. This results indicate that one more (or less) phosphate group on the protein surface does not affect greatly the partitioning of proteins with almost identical structure except the number of surface phosphoryl groups in a PEG/dextran two-phase system. Addition of small amount of metallated PEG derivatives to aqueous two-phase systems, however, can increase the partition coef-

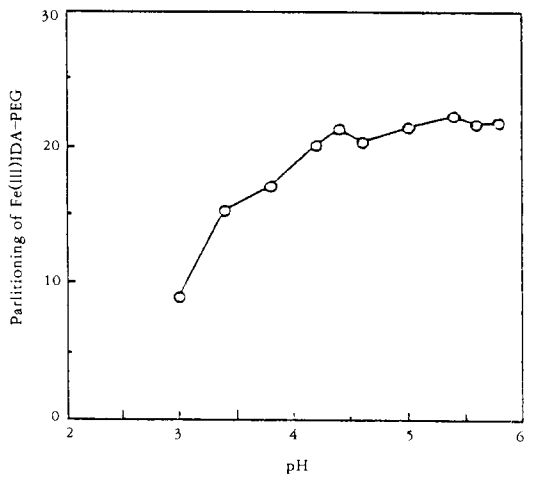

Fig. 2. Effect of pH on the partitioning of ferric ion in PEG/dextran two-phase systems. 4 % MPEG 5,000 was replaced by Fe(III)IDA-PEG.

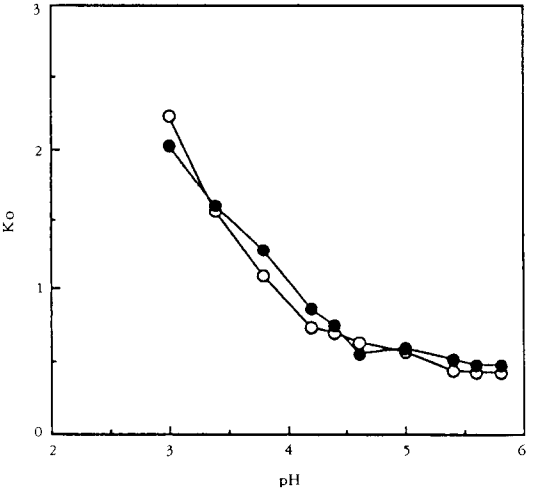

Fig. 3. Partitioning of Al(○) and A2(●) subcomponents in PEG/dextran two-phase systems containing no metallated PEG derivative.

ficients of metal binding proteins exponentially with the number of metal binding sites, as observed in the metal affinity partitioning of histidine(1,2).

The capability of metal ions to fractionate proteins by the number of metal binding sites offers a potential for application of metal-affinity partitioning technique to isolation of a high-valued protein with high purity from crude protein mixtures such as cell lysates. Multi-stage processes are generally inevitable to attain a reasonable degree of separation in aqueous two-phase extractions. Metal affinity partitioning may make multiple extraction stages reduced to one or two stage (s) by combining such fine protein fractionation capabilities of some particular metal ions and their high affinities for specific functional groups on the protein surface.

A Mathematical Model for Prediction of Phosphoprotein Partitioning

A mathematical model for metal affinity partitioning has been developed to describe protein partitioning in Cu(II)PEG/dextran two-phase systems(2). In this model the competition between the metal ligand and hydrogen ion for the surface histidine was considered, and fundamental parameters describing the average hydrogen ion affinity and metal binding affinity for surface histidine of a protein were obtained from a least-square fit of model equation to the protein partitioning data. This offers a new analytical tool for the measurement of metal-protein coordination parameters.

Most of trivalent metal ions such as Fe(III), Al (III) and Cr(III), in general, have strong affinities for hydroxyl ions compared to divalent ions (11). Binding of hydroxyl ions to metals, therefore, should be considered in the coordination between proteins and trivalent metal ions. The previous model was more extended to account for the competition between hydroxyl ions and metal binding groups (phosphoryl groups in the case of phosphoproteins) for metals.

For metal affinity aqueous two-phase partitioning of proteins which contain a two-metal chelating site (e.g. His-X_3-His in an α-helix), the following expression has been derived(12).

$$\frac{K}{K_o} = \left(\frac{(1+K_H[H])^2 + K'_a \frac{R+1}{R+1/K_M}[M_{tot}]}{(1+K_H[H])^2 + K''_a \frac{R+1}{K_M R+1}[M_{tot}]} \right)^n \quad (1)$$

Fig. 4 shows the suggested structure of the phosphoprotein/Fe(III)IDA-PEG complex. The coordination between surface accessible phosphoryl group of ovalbumin and Fe(III)IDA-PEG is assumed to be analogous to that between His-X_3-His in an α-helix and Cu(II)IDA-PEG, since the phosphoryl group has two adjacent oxygen sites which can chelate a single ferric ion. The crystal structure is not available, and thus the coordination model is not yet clear. However, the coordination structure between ferric ion and phosphoserine on the protein surface can be presumed from the binding structure between calcium or magnesium ion and L-serine orthophosphate suggested by Childs(13). If a phosphoprotein contains n moles of surface phosphoryl groups per mol of protein and two chelating oxygen sites of each phosphoryl group have different pKa values, then the eq. (1) can be modified into

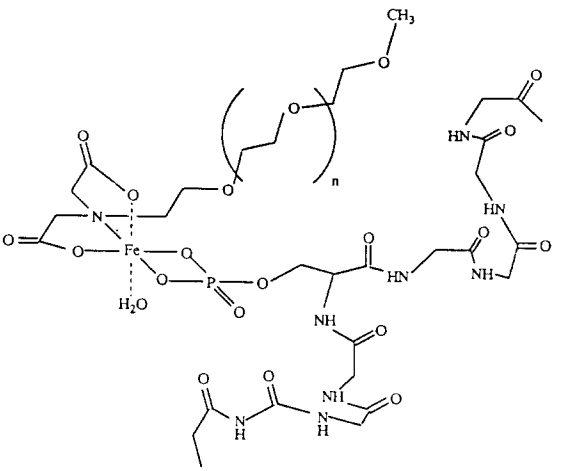

Fig. 4. Suggested structure of the phosphoprotein /Fe(III)IDA-PEG complex.

$$\frac{K}{K_0}=\left(\frac{1+K_{H1}[H]+K_{H1}[H]+K_{H1}K_{H2}[H]^2+K'_a\left(\frac{R+1}{R+1/K_M}\right)[M_{tot}]}{1+K_{H1}[H]+K_{H2}[H]+K_{H1}K_{H2}[H]^2+K''_a\left(\frac{R+1}{K_MR+1}\right)[M_{tot}]}\right)^n \quad (2)$$

At increased pH values, formation of the monodendate complexes such as PM(OH) and PM(OH)H can occur, resulting from the competitive binding between the phosphate (P) or protonated phosphate (PH) and hydroxyl ion (OH) for Fe(III)IDA-PEG (M). Assuming that formation of these monodendate complexes can be neglected compared to that of the bidendate complex (P=M), total polymer-metal concentration, [Mtot], in eq.(2) can be replaced by the concentration of polymer-metal ligand, [M], unbound to hydroxyl ions which can form the bidendate complex.

$$[M]=\frac{[M_{tot}]}{1+K_{OH}[OH]} \quad (3)$$

The assumption made above can be justified by the previous report(12). The affinities for formation of the multidendate complexes are much higher than those for formation of the monodendate complexes. The stability constant for formation of the complex between His-X_3-His site in an α-helix and Cu(II)IDA-PEG is more than ten times the stability constant for interaction of Cu(II)IDA-PEG with two independent histidines. The pKa values for the first ionization of the phosphoryl group of a variety of phosphate compounds are much less than 1.0 (13), so that $K_{H1}[H]$ in eqn (3), the hydrogen association constant (K_{H1}) for the first ionization of two oxygen chelating sites times hydrogen ion conentration ([H]), becomes approximately zero in the pH range of interest. Combining of eqs. (2) and (3) yields

$$\frac{K}{K_0}=\left(\frac{1+K_{H2}[H]+K'_a\left(\frac{R+1}{R+1/K_M}\right)\frac{[M_{tot}]}{1+K_{OH}[OH]}}{1+K_{H2}[H]+K''_a\left(\frac{R+1}{K_MR+1}\right)\frac{[M_{tot}]}{1+K_{OH}[OH]}}\right)^n \quad (4)$$

where K and K_0 are the partition coefficients in the presence and absence of the metal chelate, respectively, R is the phase volume ratio, K_M is the partition coefficient of the polymer-metal chelate, n is the number of binding sites, [OH] is the hydroxide ion concentration, and $[M_{tot}]$ is the total metal concentration in the two-phase system. As previously defined(2), K_a, K_H and K_{OH} denote the association constants for the competing reactions of each species, where the subscripts a, H and OH represent metal, hydrogen and hydroxide ions, respectively. These association constants in eq. (4) were evaluated by fitting the model equation (4) to the partitioning experimental data of A2 with one phosphoryl group using the nonlinear regression analysis. The resulting values are $K_a'=6.1\times10^3$, $Ka''=2.3\times10^4$, $K_{H2}=1.1\times10^4$, and $K_{OH}=6.3\times10^8$, or log $K_a'=3.8$, log $K_a''=4.4$, log $K_{H2}=4.0$, and log $K_{OH}=8.8$.

The binding constants for formation of the complex between the phosphoryl group on the protein surface and the Fe(III)IDA-PEG are 3-5 times those for interation of Cu(II)IDA-PEG with a single surface-accessible histidine(2). The resulting pKa value for the second ionization of the phosphoryl group on the surface of ovalbumin is 4.0, which is much smaller than the apparent pKa values (5.5~6.0) of many phosphorylated compounds obtained by the titration method(10). The reason for this remains unknown. The pKa values can be affected by environmental factors such as electrostatic forces. Metal ions might be able to alter the pK values of surface amino acids of a protein. The pK_{OH} value (=8.8) for formation of the complex between hydroxyl ion and Fe(III)IDA-PEG is much smaller than that (pK_{OH}=11.1) for interaction of hydroxyl ion with free ferric ion(11).

As shown in Fig. 5, a good agreement was observed between theoretical predictions and experimental data obtained with A2. Using these values, the partitioning data of A1 with two phosphate groups were predicted theoretically, but the discrepances were observed especially at decreased pH values. This seems to be caused by oversimplifications made for deriving eq. (4). A

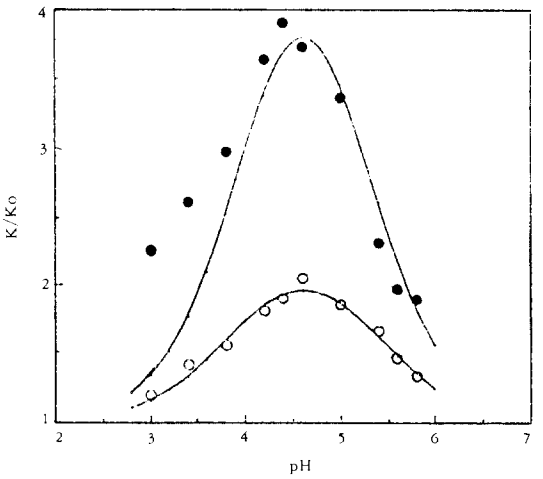

Fig. 5. Plots of pH vs K/Ko:(●)A1;(○)A2. Solid lines represent theoretical predictions.

better fit might be obtained if the association constants are assigned to each binding site. Above pH 4.6, however, the experimental data of A1 are well-described by the model equation. This pH dependence can be explained by arising from deprotonation of the second oxygen of the phosphoryl group and the competitive binding of hydroxyl ions to the metal ion.

In conclusion, partitioning of phosphoproteins can be enhanced by the addition of Fe(III)IDA-PEG ligand to a PEG/dextran two-phase system, due to interaction of the phosphoryl group on the protein surface with ferric ion. The partitioning behavior was well-described by the model equation which takes into account inhibition of ligand binding by both hydrogen and hydroxyl ions. This model may be used to describe the partitioning behavior of proteins in the metal affinity aqueous two-phase systems where metal ions attached to PEG derivative have strong affinities for hydroxyl ions as well as particular functional groups on the protein surface.

요 약

Fe(III)IDA-PEG/dextran 액 이상 분계 시스템에서 phosphoprotein의 분배 예측을 위한 모델식이 유도되었다. 본 모델식에는 단백질 분배에 수소이온과 hydroxide 이온의 저해효과가 포함되어 있다. 표면에 각각 1,2개의 phosphoryl 그룹을 갖고 있는 ovalbumin의 A1, A2, 성분을 정제한 후 단백질 분배 실험을 수행하였으며 실험결과와 모델식에의한 예측이 잘 일치하는 것을 확인하였다. Fe(III)IDA-PEG와 단백질 표면 phosphoryl group과의 결합상수는 PEG 상과 dextran상에서 각각 $6.1\times10^{3}M^{-1}$, $2.3\times10^{4}M^{-1}$이었으며,이것은 Cu(II)IDA-PEG와 단백질 표면 histidine과의 결합상수 보다 3-5배 높은 값이었다.

NOMENCLATURE

[H]	hydrogen ion concentration (M^{-1})
K	protein partition coefficient in the presence of ligand
K_a	binding constant for protein-ligand complex (M^{-1})
K_H	hydrogen ion association constant (M^{-1})
K_{H1}	hydrogen ion association constant for the first ionization of the phosphoryl group (M^{-1})
K_{H2}	hydrogen ion association constant for the second ionization of the phosphoryl group (M^{-1})
K_M	partition coefficient of polymer-metal affinity ligand
K_o	protein partition coefficient in the absence of ligand
K_{OH}	hydroxyl ion association constant for Fe(III)IDA-PEG (M^{-1})
[M]	concentration of polymer-metal ligand unbound to hydroxyl ions (M)
$[M_{tot}]$	total metal concentration (M)
n	number of binding sites
[OH]	hydroxide ion concentration (M)
R	volume ratio of top and bottom phases

Superscripts

′	top phase
″	bottom phase

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