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METACHROMASY OF CRYSTAL VIOLET IN THE PRESENCE OF POLY(α-L-GLUTAMIC ACID) AND THE BOUND-DYE SPECTRA DETERMINED BY THE PRINCIPAL-COMPONENT-ANALYSIS METHOD*

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In order to study quantitatively the metachromatic behaviour of crystal violet (CV) in the presence of poly(α-L-glutamic acid), (poly(Glu)), four sets of the absorption spectra of the poly(Glu)-CV system were analyzed by the extended principal-component-analysis (PCA) method. Two classes of CV-Glu complexes, i.e., the bound-CV species, are present in poly(Glu) regardless of its helical and random-coiled conformations over a wide range of the mixing ratios of Glu residues to CV (P/D). The spectra of the bound CV in a low P/D range < 100 (complex I), extracted by the PCA method, are conformation-dependent showing three absorption bands at 506, ca. 550, and 610–620 nm. The spectra of the bound CV in a high P/D range > 100 (complex II) are closely related to, but not identical with, the free CV. The molar fractions of free CV and complexes I and II, evaluated in the P/D range of 0–150, indicate that CV binds more to the random-coiled poly(Glu) than to the helical one. Metachromasy of CV results from a complicated interplay of an unbound and two differently bound species.

1. Introduction

Metachromasy, the well-known phenomenon of spectral changes of a dye in the presence of a polyelectrolyte, has been investigated on the various dye-polyelectrolyte systems [1–22]. In some of these investigations, attempts were made to extract the pure spectrum or spectra of the bound dye from a series of experimental spectra of the dye-polyion system by the equilibrium dialysis method [4–6,19], the graphical method [7,17], or the extended principal-component-analysis (PCA) method [20,22]. The numbers of the chemical species of dye bound to polyion and the corresponding bound-dye spectra are of utmost importance in the precise and quantitative interpretation of metachromasy.

The PCA method was previously extended to cover chemical equilibria [23] in such a way that the analysis of the metachromatic behaviour of a dye-polyion system becomes possible. The extended PCA method treats such spectra as absorption, circular dichroism, and optical rotatory dispersion, as the vectors [20,23, 24], and requires no assumption except for an appropriate equilibrium scheme. Therefore, this computer-based method can process the experimental data, collected without disturbing an equilibrium system, to extract information not only on the number of the colored species (absorbers) in a chemical equilibrium but also on the unknown spectra of the species and the equilibrium constant. In equilibrium dialysis [4–6, 19], for example, the measurement of the equilibrium concentrations is required, but the dialysis membrane, which adsorbs dye molecules, is a major disturber and the Donnan effect is another serious problem. If this effect is suppressed by the addition of a simple electrolyte, a competitive reaction often occurs between the added salt and the ionic dye to shift the true equilibrium condition.

Metachromasy resulting from the interaction between dye and polyelectrolyte is affected by a particular conformation of the polymer besides pH, temperature, ionic strength, and mixing ratio of polymer residues to dye (P/D). Attempts have been initiated

* This is Part VI of Metachromasy. For the preceding paper, see ref. [22].

for the differentiation of the polymer conformation by the use of a metachromatic dye [6,15,19,21]. For this purpose, poly(amino acids), which undergo reversible conformational changes by experimental conditions, are suited for the model system. Poly(α-L-glumatic acid) has been extensively used in combinations with dyes of various structures which show some pronounced metachromasy bands in the visible absorption spectra [1,2,10,11,16,18,21].

In this work, the extended PCA method has been applied to the systems between crystal violet (CV), which shows a remarkable metachromasy [3,8,9,11,13, 20,22], and poly(α-L-glutamic acid), which retains either random-coiled or helical conformation, over a wide P/D range Free CV and a kind of bound CV are present in a low P/D range, while two kinds of bound CV (designated as complexes I and II) are formed in a high P/D range. The unknown pure spectrum and the binding curve of each species are given; the spectra of the bound CV in a neutral pH differ from those in an acid pH showing the conformation-specificity of CV.

2. Experimental

2.1. Materials

Sodium poly(α-L-glutamate), hereafter denoted simply as poly(Glu), was a gift of Dr. Hiroshi Sato of Mitsubishi Rayon Co. It was purified by fractional precipitation from the water-acetone system. The weight-average degree of polymerization was ca. 590, which was estimated from an intrinsic viscosity of 0.747. Crystal violet in the form of chloride was purchased from Chroma Gesellschaft, Schmid & Co. and was purified by recrystallization [13].

2.2. Procedures

The following four poly(Glu)-CV systems A-D were studied: in systems A and B the pH was adjusted to about 7.4 and 5.0 with sodium cacodylate buffer respectively. The pH was about 7.6 in system C, while it was adjusted to 4.1 by adding HCl in system D. The concentration of CV was fixed at ca. 1×10^{-5} mol/dm^3 in all systems. Each sample solution was prepared for absorption measurements separately. The extended PCA procedure was applied to a family of five to seven absorption spectra for each poly(Glu)-CV system in the same manner as already described elsewhere [20, 22]. A summary of the present procedure is given in Appendix.

2 3. Measurements

Absorption spectra were measured with a Hitachi model EPS-3T recording spectrophotometer at 25°C. Matched quartz cells of 1 cm in path length were used. The pH of sample solutions was measured on a Hitachi-Horiba model F-7 pH meter with a compound electrode.

3. Results and discussion

3 1. Low P/D range systems A and B

The observed spectra in systems A (P/D = 0—1.0) and B (P/D = 0—2.0) and the pure spectra of CV bound to poly(Glu), extracted by the extended PCA method, are shown in fig 1. The experimental spectra in both systems have a broad maximum at 592 nm and two isosbestic points at 530 and 636 nm for system A and at 528 and 633 nm for system B as indicated by arrows. The existence of these isosbestic points in each family of the spectra indicates that each system may be composed of only two colored components, probably a free CVa and bound CV, and that the bound CV may depend on the conformation of poly-(Glu). The absorbance at the metachromasy band (506 nm) increases with an increase in P/D values, indicating that the pure spectrum of each bound-CV species may have a major absorption peak near 506 nm.

The PCA procedure was applied to each family of the observed spectra in order to determine the number of the colored species. The eigenvalues Λ_i were plotted against components on a logarithmic scale in fig. 2. In each system a large gap is clearly seen between the eigenvalues of the second and the third component, while the differences in eigenvalues between the third and the higher components are small and the eigenvalue of the third is almost the same as the noise level. The value of $\log(\Lambda_1/\Lambda_3)$ was about 5—6, which is beyond the present criterion (the dashed line). Therefore, it can be concluded that these systems contain only two colored components [20,22,23]: one is the free CV and the other is the CV bound to poly(Glu).

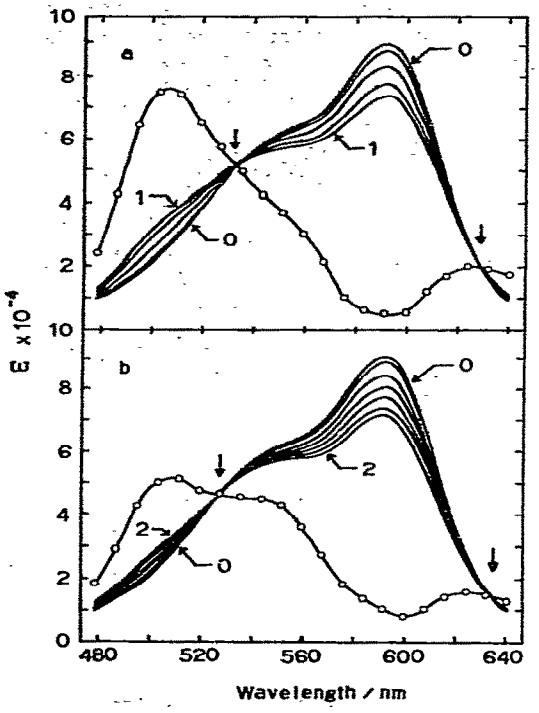

Fig. 1. Absorption spectra of CV in the presence and the absence of poly(Glu) and the pure absorption spectra of bound CV (circles) in the low P/D range. (a) system A (pH = 7.4) and (b) system B (pH = 5.0). The P/D values are given in terms of the molar ratio of Glu residues-to-CV in each poly(Glu)-CV solution. The experimental molar absorption coefficient ϵ (mol^{-1} cm^{-1} dm^3) is expressed in terms of the total CV concentration in each solution, while the ϵ-value of bound CV is computed from the ϵ-value of all the solutions with the aid of eqs. (4) and (5) of ref. [20]. The P/D values are 0, 0.2, 0.4, 0.6, and 1.0 for (a) and 0, 0.2, 0.6, 1.0, 1.4, 1.8, and 2.0 for (b), as indicated.

Since the binding reaction of a polyion with a dye cation often deviates from a simple equilibrium scheme, the previous equilibrium equation with a single adjustable parameter, α, [20,22] will be used here for the PCA procedure:

$$K_1 = [DP^*]/[D][P]^{\alpha} \quad (1)$$

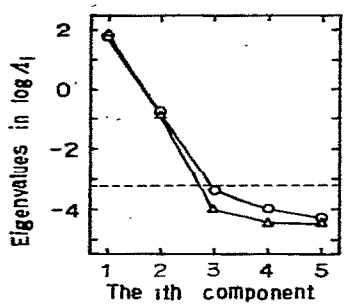

Fig. 2. Relation between eigenvalue Λ_i and the ith component for system A (circles) and system B (triangles).

where D and P are the free CV and the unoccupied binding site of poly(Glu), respectively, while DP^* is the bound CV which is associated with an absorption spectrum different from that of the free CV. The brackets indicate the equilibrium concentration of each species. The α is an empirical parameter, which is affected by many factors such as temperature, ionic strength, added salt, P/D, and the degree of polymerization of a polymer [22], and is determined by optimizing the deviation of the equilibrium constants from the mean value [20,22].

The mean equilibrium constant and the optimized α value are given in table 1. The K_1' in the table is a quantity related to the K_1 as:

$$K_1' = K_1[P]^{\alpha-1} \quad (2)$$

and is equal to $[DP^*]/[D] \cdot [P]$ at P/D = 1 [20,22]. These K_1' values suggest that the binding of CV to poly(Glu) is much weaker than the binding to most of other polyelectrolytes [20]. The result also indicates

Table 1
The empirical parameter α and the equilibrium constants K_1 and K_1' in the low P/D range

Systems	α	K_1 a)	K_1' b)
A (pH = 7.4)	2.0	9.2×10^9	4.3×10^4
B (pH = 5.0)	1.3	4.8×10^5	1.2×10^4

a) The dimension is given by $[mol \cdot dm^{-3}]^{-\alpha}$.
b) $K_1' = K \cdot [P]^{\alpha-1}$ at P/D = 1. The dimension is given by $[mol \cdot dm^{-3}]^{-1}$.

that CV binds less to the helical poly(Glu) than to the random-coiled poly(Glu). If the α value in eq. (1) is less than, or equal to, unity, the optical titration curve of a free CV solution with a poly(Glu) solution should be monotonous [20]. However, the α values for systems A and B are both larger than unity, suggesting that the titration curve would have a convex curvature in the initial low P/D range.

The pure spectra of bound CV in both systems are shown by open circles in fig. 1. Three metachromasy bands are clearly discernible. They are termed the Meta S_1, Meta S_2, and Meta L bands (S and L signify the bands on the shorter- and longer-wavelength sides of the principal band of the free cationic CV with the suffix number from the one closest to it) [20]. The bound-CV spectra have a major absorption band (the Meta S_2) at 506 nm and a distinct shoulder (the Meta S_1) near 550 nm. The positions of the Meta L band, however, differ from each other in systems A and B. This difference may result from the conformation of poly(Glu), as was already observed in the DNA-CV systems [20]. The bound-CV spectra in systems A and B are similar to those obtained in many polyelectrolytes with various ionizable groups [20]. Nevertheless, subtle differences in the bound spectra are clearly noted. They depend on the conformations and the side chain groups of individual polyelectrolytes. This specificity seems to be particularly reflected on the absorption intensity of the Meta S_1 band near 550 nm (cf. fig. 6 of ref. [20]).

3.2. High P/D range systems C and D

The observed spectra in systems C (P/D = 60–150) and D (P/D = 20–75) are shown in fig. 3. With the increase in P/D, the absorbance at the band maximum of free CV (592 nm) increases, while the absorbance at the metachromasy band (506 nm) decreases. The spectra in these two systems seem to have three bands, respectively, but their relative intensities differ depending on the conformation of poly(Glu). The isosbestic points indicated by arrows (530 and 638 nm in fig. 3a, and 536 and 647 nm in fig. 3b) in each family of the spectra suggest that both systems are binary containing probably two kinds of the CV-Glu complexes, i.e., two bound-CV species.

The eigenvalues are plotted against the components in fig. 4. The number of the colored species in each sys-

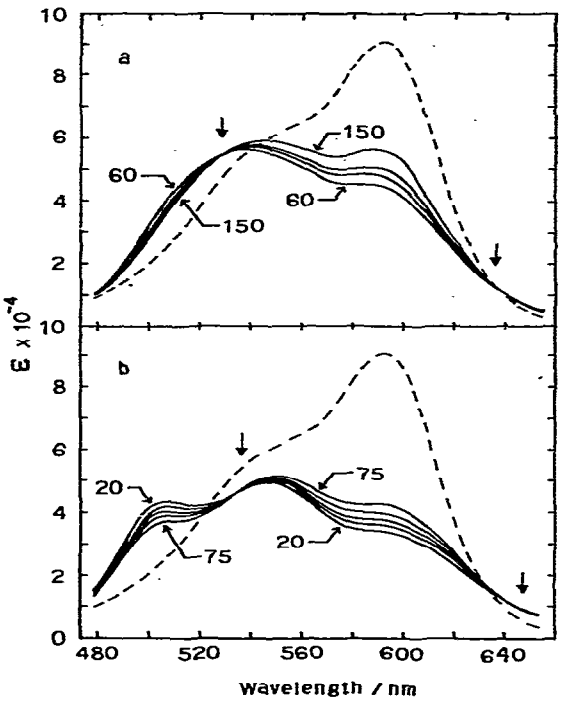

Fig. 3. Absorption spectra of CV in the presence of poly(Glu) in the high P/D range (solid curves) together with the spectra of CV only (dashed curves). (a) system C (pH = 7.6) and (b) system D (pH = 4.1). The P/D values are 60, 80, 100, and 150 for (a) and 20, 30, 40, 50, and 75 for (b), as indicated. The ϵ-values are expressed in the same manner as in fig. 1.

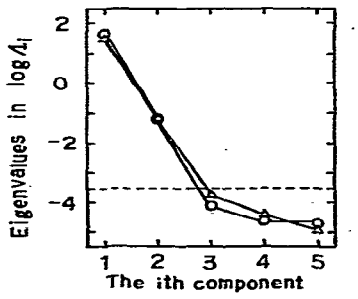

Fig. 4. Relation between eigenvalue Λ_i and the ith component for system C (circles) and system D (triangles).

tem is indeed determined to be two from the relative magnitude of the eigenvalues. It was concluded that these two species in each system were the bound CV, without the free CV, in contrast to the low P/D range systems in which one is the free CV and the other is the bound CV. This conclusion was based on the result that the addition of the absorption spectrum of the free CV made the eigenvalue of the third component, Λ_3, larger than the preset limit.

Now, the two bound-CV species and their spectra are all unknown *a priori* and the binding reactions between CV and poly(Glu) in a high P/D range have not been established experimentally. Therefore, a number of equilibrium schemes were tested in this work for the PCA procedure, in order to optimize the deviation of the computed equilibrium constants and to extract the bound spectra. After several trial-and-error tests, the following two types of schemes were found satisfactory:

$$K_2 = [\mathrm{DP}]^{\beta}/[(\mathrm{PD}^*)_2]\, P_t, \tag{3}$$

and

$$K_3 = [\mathrm{DP}]\,[\mathrm{PNa}]/[\mathrm{DP}^*\mathrm{Na}^+][\mathrm{P}^-]^{\gamma}. \tag{4}$$

Eq. (3) is based on the analogy with the dimer formation of two nearest-neighboring dyes, each being bound to a Glu site. DP and $(DP^*)_2$ may thus be a bound monomer and a bound dimer, and P_t is the total residue concentration of poly(Glu) in a sample solution. If the dimer is actually formed, the value of β should probably be two. Eq. (4) represents a case in which a nonabsorbing component is involved. DP and PNa are the bound dye and the neutralized binding site. P^- and DP^*Na^+ symbolize the dissociated site and the dye bound to the site in which an Na^+ (nonabsorber) is involved. The β and γ are presently considered only as the empirical parameters and may include such various factors as the shape factor, electrostatic potential, and others characteristic of a polyelectrolyte. Neither of schemes (3) and (4) is claimed to be unique at present.

The PCA results are given in table 2. Both β and γ would be two and one, respectively, if a simple dimer formation or a participation of counterions occurred on a poly(Glu) chain. This result indicates that the true binding reaction may be more complicated than that expressed by either eq. (3) or (4). For convenience's sake, each of components $(DP^*)_2$ and DP^*Na^+ will be termed complex I, which is outstanding on a lower P/D side, while the other component DP will be named complex II, which is predominant on a higher P/D side. The pure spectra of complexes I and II in system C are shown in fig. 5(a). The spectra of complexes I obtained on the basis of the two assumed equilibrium schemes agree well. The difference in terms of the ϵ-value is less than ±4% between the two schemes; the same is true of complexes II. The spectrum of bound CV in complex I shows the Meta L and Meta S_1 and S_2 bands, while the one in complex II is rather close to, but by no means identical with, the spectrum of free CV.

The pure spectra of complexes I and II in system D are shown in fig. 5(b). They depend slightly on the equilibrium schemes. The spectrum of complex I now shows a distinct Meta S_1 peak at 545 nm and the Meta L band at 610 nm, while that of complex II is quite different from the spectrum of the free CV. Comparison of the spectrum of complex I in system C (fig. 5a) with the bound-CV spectrum in system A (fig. 1a) reveals that these absorption spectra are very alike, showing peaks at ca. 510 and 620 nm and a trough near 600 nm. The bound-CV spectrum in system B (fig. 1b) is also closely related with the spectrum of complex I in system D (fig. 5b), both having three metachromasy bands near 510, 550, and 610–620 nm.

From the above results, the bound CV in the low P/D range may be considered to belong to the same class as complex I (both of them will be denoted simply as complex I), as far as the conformation of poly(Glu) remains almost unchanged in the neutral or acid pH range. Some minor differences in the spectral pro-

Table 2
The empirical parameters β and γ and the equilibrium constants K_2 and K_3 for the poly(Glu)-CV solutions in the high P/D range

Systems	β	K_2 [a]	γ	K_3 [b]
C (pH = 7.6)	2.3 (1%) [c]	7.1×10^{-2}	0.9 (0.94%)	8.4×10^{-3}
D (pH = 4.1)	2.9 (3.6%)	3.0	0.8 (3%)	1.5×10^{-3}

a) The dimension is given by $[\mathrm{mol \cdot dm^{-3}}]^{\beta-2}$.
b) The dimension is given by $[\mathrm{mol \cdot dm^{-3}}]^{1-\gamma}$.
c) The numeral in parentheses is the deviation of either K_2 or K_3 from the mean value.

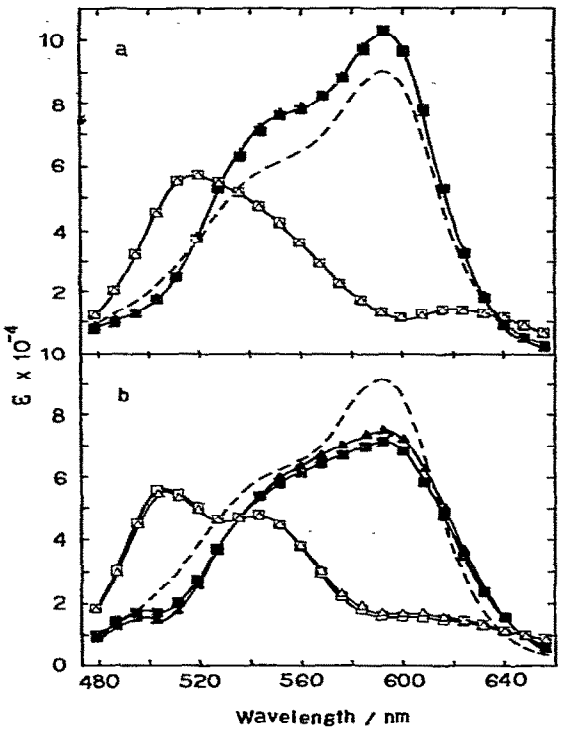

Fig. 5. Pure absorption spectra of bound CV-Glu complex I (open symbols) and bound CV-Glu complex II (closed symbols) in the high P/D range. (a) system C and (b) system D. Triangles and squares denote the spectra which were extracted by the extended PCA method with the aid of eqs. (3) and (4), respectively, while the dashed curves are the spectra of free CV for comparison. The molar absorption coefficients, ϵ, of the bound CV species are expressed in the same manner as in fig. 1.

files may be due to the solution properties such as the slightly different pH values, i.e., 5.0 versus 4.1 and 7.4 versus 7.6 with and without buffer salt. Therefore, the species responsible for metachromasy of CV bound to poly(Glu) in systems C and D is not complex II, which is dominant in the extremely high P/D range (> 100), but is complex I, which is in abundance in the relatively low P/D range (< 100). The bound-CV spectra clearly reveal that metachromatic features depend on the conformation of poly(Glu). They also verify that the spectral profiles in the very high P/D range result from the bound CV but not from the free, unbound CV.

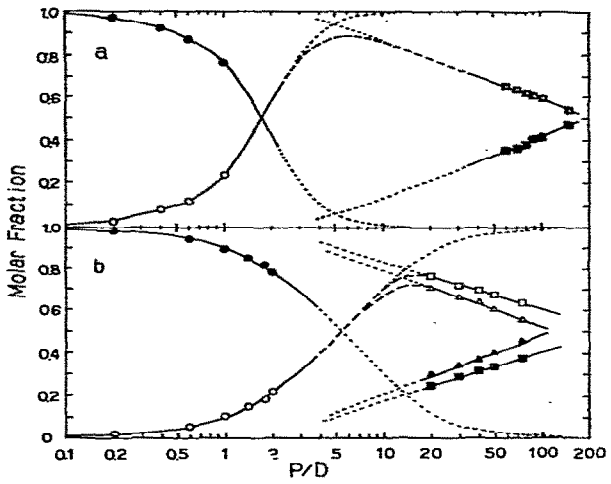

Fig. 6. The molar fraction versus P/D curves of free CV (closed circles), complex I (open symbols), and complex II (closed squares and triangles). These symbols denote the molar fractions which were obtained by the extended PCA method with the equilibrium schemes (1), (3), and (4) given in the text. Triangles and squares correspond to schemes (3) and (4) in the high P/D range, respectively, while the solid and dotted lines were obtained by the use of eqs. (1), (3), and (4) together with the values of α, β, γ, K_1, K_2, and K_3 given in tables 1 and 2, as appropriate. (a) the neutral pH system A and C, and (b) the acid pH systems B and D.

3.3. Binding curves

Fig. 6(a) shows the variation with P/D of the molar fractions of free CV (X_f), complex I (X_I) and complex II (X_{II}) in the neutral pH region which were calculated on the basis of the PCA procedure [cf. eq. (4) of ref. [22]]. The analytically obtained points are in good agreement with each sigmoidal curve (solid and dotted lines) which is calculated from the α value and the equilibrium constant K [cf. eq. (2) of ref. [22]]. The results show that X_I increases at the expense of X_f, i.e., the free CV is outnumbered by complex I at P/D = 1.8 and essentially disappears at P/D = 10. (The bound CV in the low P/D (0–1) is assumed the same species as complex I.) The X_I then decreases gradually with the increase in P/D through a maximum at a P/D of about 7 (dashed line). The X_{II} increases in the higher P/D range, as the X_I decreases; they may be equal at a P/D about 200, beyond which complex II

would be in excess of complex I, i.e., metachromasy of a poly(Glu)-CV solution begins to diminish.

The dependence of the molar fractions of X_f, X_I, and X_{II} on P/D in the acid pH region is shown in fig. 6(b). These binding curves indicate that the fraction of bound CV (X_I) at P/D = 1 is only one-tenth of the total CV present in solutions at pH 5.0, in a marked contrast to the results in fig. 6a in which the X_I in solutions at pH 7.4 is about 0.25, i.e., every fourth Glu residue is occupied by a CV. This is partly because the ionized residues are very few at the low pH. If only two colored species, i.e., a free CV and a bound CV, should prevail over an entire P/D range, the free CV would still remain at P/D = 100 in the acid pH region. However, as a second bound-CV species appears in a high P/D range, the X_f may decrease more readily disappearing in a much lower P/D range. This notion is supported by the result that only two bound-CV species, without free CV, exist in system D over a P/D range of 20–75. The molar fraction versus P/D curves of the poly(Glu)-CV complexes obtained from the PCA procedure differ slightly according to the equilibrium scheme, (3) or (4). Nevertheless, they show almost the same tendency.

3.4. Closing remarks

The extended PCA method has been shown to be useful for quantitative analysis of the metachromatic behaviour of CV bound to poly(Glu) in the helical or in the random-coiled conformation. It is worth mention that only two classes of the bound-CV species are sufficient to describe the seemingly complicated spectral changes over a wide P/D range. The corresponding bound-CV spectra could also be extracted from a reasonable number of experimental spectra according to the PCA procedure for which binding reaction schemes (1), (3), and (4) were utilized. Refinement of the extended PCA method is highly desirable; for example, these schemes must be considered only empirical at present and subject to a further scrutiny, and the physical meaning of the parameters α, β, and γ also remains for clarification. The PCA analysis should be applied to the experimental data available in an intermediate P/D range ($2 \leqslant P/D \leqslant 50$) in which probably three colored species are coexisting (free CV and two CV-Glu complexes), once some appropriate equilibrium schemes are devised for such ternary systems.

Acknowledgement

We thank Mr. Kiyoshi Mitsuda for furnishing the absorption spectra of poly(Glu)-CV systems which made the present work possible and Dr. Mineo Takatsuki for his constructive advice.

Appendix

Outline of the extended PCA method

The procedure of the extended PCA method will be outlined here. (For details, see refs. [20, 22–24].) Consider a system in chemical equilibrium containing p light-absorbing components. According to Beer's law, a data matrix, **D**, which is composed of the absorbances at n wavelengths for m experimental spectra, is expressed as:

$$\mathbf{D} = \mathbf{C}\boldsymbol{\varepsilon} = \begin{pmatrix} C_{11} \cdots C_{1p} \\ \cdots\cdots\cdots \\ C_{m1} \cdots C_{mp} \end{pmatrix} \begin{pmatrix} \epsilon_{11} \cdots \epsilon_{1n} \\ \cdots\cdots\cdots \\ \epsilon_{p1} \cdots \epsilon_{pn} \end{pmatrix}, \qquad (A.1)$$

where **C** is a concentration matrix and $\boldsymbol{\varepsilon}$ is a molar-absorption-coefficient matrix.

The second-moment matrix, **A**, is constructed from the data matrix and its transposed matrix as follows:

$$\mathbf{A} = {}^{t}\mathbf{D}\mathbf{D}. \qquad (A.2)$$

The eigenvalues and the corresponding eigenvectors are obtained by diagonalizing the **A** matrix. The number of the significant components (i.e., p) can be determined from the relative magnitude of the eigenvalues [20, 22–24], since the p eigenvalues are much larger than the others or than a noise level in a real system.

Next, a linear-combination-coefficient matrix, **f**, and a transformation matrix, **t**, are introduced as follows:

$$\mathbf{D} = \mathbf{fe}, \qquad (A.3)$$

and:

$$\boldsymbol{\varepsilon} = \mathbf{te}. \qquad (A.4)$$

The **e** matrix, which consists of p eigenvectors, is transformed to the **D** matrix by the **f** matrix, and also to the $\boldsymbol{\varepsilon}$ matrix by the **t** matrix. The **C** matrix is obtained as:

$$\mathbf{C} = \mathbf{f}\mathbf{t}^{-1}. \tag{A.5}$$

The pure spectra of the p unknown components and their concentrations involved in each data spectrum can be evaluated, once the t matrix is available.

In order to determine the t matrix, an appropriate equilibrium scheme must be selected [20, 22–24]. The general expression for the scheme may be written as:

$$K = \prod_{i=1}^{q} [X_i]^{\nu_i}, \tag{A.6}$$

where K is the equilibrium constant, X_i ($i = 1, q (q \geqslant p)$) and ν_i are the ith component and its stoichiometric coefficient, respectively. The brackets indicate the equilibrium concentration. The quantity of S (A.7) is the criterion for selecting the most appropriate scheme for which the value of S reaches the minimum [20,23, 24].

$$S = \left[\frac{1}{m} \sum_{j=1}^{m} (K_k - \bar{K})^2\right]^{1/2} / \bar{K}, \tag{A.7}$$

where $\bar{K}$ is the mean equilibrium constant averaged over m equilibrium constants (K_j ($j = 1, m$)). The t matrix should be determined by iterative calculations.

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