Interaction of Ribonuclease A with Aqueous 2-Methyl-2,4-pentanediol at pH 5.8†

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ABSTRACT: The interactions between ribonuclease A and solvent components in aqueous 2-methyl-2,4-pentanediol (MPD) have been investigated by differential refractometry and light scattering at pH 5.8, i.e., conditions similar to those used to crystallize the protein from this solvent system. Application of multicomponent thermodynamic theory shows that, at all solvent compositions up to 50% (v/v) MPD, the protein is preferentially hydrated; i.e., addition of ribonuclease to the mixed solvent leads to an increase in the chemical potential of MPD. This unfavorable thermodynamic interaction leads to phase separation, probably caused by local salting out of the MPD by the charges on the surface of the protein molecule. A parallel examination by circular dichroism (CD) has shown that the CD spectrum of ribonuclease in 50% MPD is indistinguishable from that in dilute buffer.

Although the most detailed information on the three-dimensional structure of protein molecules is obtained from x-ray crystallographic analysis, the absolute validity of the extrapolation to dilute solutions of the structural details observed in the crystal state has, at times, been questioned. This question may become highly significant if the space coordinates of the residues in an active site were not fully identical in the crystalline state and in the protein dispersed at concentration levels typically used in enzyme activity studies. Because of small redistributions of the free energy of conformational stability of surface residues, crystallization may induce displacements of amino acid residues at points of contact between individual protein molecules within the crystal. The existence of such contacts involving active site residues is well documented. For example, in the case of α-chymotrypsin, it is known that the crystal state asymmetric unit is a dimer with intermolecular contacts involving active site residues, in particular histidine-57 (Sigler et al., 1968; Timasheff, 1970; Aune and Timasheff, 1971; Aune et al., 1971).

The use of unnatural media, such as concentrated salts or organic solvents, in the crystallization of proteins also introduces the possibility of structural alterations as a result of solvent effects on the local free energy of conformational stability of amino acid residues exposed to the surface (Perutz and Ten Eyck, 1971). In fact, recently several investigators have questioned the identity of conformations of individual proteins in the crystalline and solution states. Particularly striking are the conclusions reached by Johansen and Vallee (1971, 1975) that the crystallization of carboxypeptidase A leads to the displacement of tyrosine-248 and that of Winstead and Wold (1965) that exposure of ribonuclease A to ammonium sulfate (the crystallization medium for ribonuclease S) results in an increase in the specific activity of the enzyme, which these authors have attributed to a conformational change. There is also evidence that α-chymotrypsin dimers seen in solution and in the crystal differ in details of packing (Tulinsky et al., 1973; Aune and Timasheff, 1971).

A particularly intriguing medium, used to crystallize ribonuclease A (King et al., 1956) and staphylococcal nuclease (Cotton et al., 1966), is aqueous 2-methyl-2,4-pentanediol (MPD).[1] The water–MPD solvent system can be expected to have a partially hydrophobic character, since the MPD molecule contains six methylene groups, and one might expect it to destabilize the native structure of a protein. Indeed, Scherage and co-workers (Schrier and Scheraga, 1962; Schrier et al., 1965) have shown that alcohols lower the transition temperature of ribonuclease A, a reflection of their destabilizing action on the native structure. Alcohols in general are known to favor denaturation or precipitation of proteins at room temperature (Herskovits et al., 1970). Furthermore, solubility measurements on amino acids in 55% MPD, the crystallizing medium for ribonuclease A, have shown a favorable free energy of transfer of most amino acid side chains to this medium (Pittz and Bello, 1971), suggesting that it should have a denaturing, rather than crystallizing action. On the other hand, in their examination of the effect of 50% MPD on the native structure of RNase A, Pittz and Bello (1973) have reported that the CD spectra of RNase A in water and 50% MPD were indistinguishable down to 210 nm.

In view of the importance of the questions of the integrity of protein structure in crystallizing media, and of the solvent interactions which favor protein crystallization, we have undertaken a detailed examination of such systems in solution. The approach selected was that of parallel measurements of the thermodynamic interactions between the protein and components of the solvent system, and of possible conformational changes as detected by circular dichroism. It is the purpose of this paper to report on studies of bovine pancreatic ribonuclease A in the water–MPD system at conditions similar to those used in the crystallization of this protein for the structural studies.

Experimental Procedures

Materials. Ribonuclease A (bovine pancreatic, 5× crystallized, EC 3.1.4.22) was purchased from Sigma Chemical

† Publication No. 1186 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received June 6, 1977; revised manuscript received October 21, 1977.* This work was supported in part by National Institutes of Health Grants No. GM-14603 and CA-16707 and National Science Foundation Grant No. PCM72-02572.

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[1] Abbreviations used: MPD, 2-methyl-2,4-pentanediol; RNase A, ribonuclease A (bovine pancreatic, 5× crystallized, EC 3.1.4.22); ATA, *N*-acetyl-L-tyrosinamide; CD, circular dichroism.

0006-2960/78/0417-0615$01.00/0

TABLE I: Partial Specific Volumes of Water ($\bar{v}_1$) and 2-Methyl-2,4-pentanediol ($\bar{v}_3$) in Binary Mixtures (pH 5.8, 25 °C, 0.01 M Sodium Acetate, 0.02 M NaCl).

MPD (vol %)	Density (g/mL)	$\bar{v}_1$ (mL/g)	$\bar{v}_3$ (mL/g)
0	0.99859		
10	0.99810	1.0020	1.0020
20	0.99850	1.0030	0.9955
30	0.99675	0.9965	1.0225
40	0.99419	0.9810	1.0480
50	0.98705	0.9765	1.0565
60	0.97852	0.9755	1.0575
70	0.96836	0.9740	1.0740

Co., St. Louis, Mo. *N*-Acetyl-L-tyrosinamide was purchased from Mann Research Labs., New York, N.Y. Sodium acetate and sodium chloride were reagent grade. MPD was purified by the method of Bello and Nowoswiat (1965); 2-chloroethanol was redistilled prior to use. All water used in experiments was glass distilled and passed through deionizing columns before use. For light-scattering experiments, the water was further clarified to remove dust particles (Timasheff and Townend, 1970; Pittz et al., 1975).

All protein and *N*-acetyl-L-tyrosinamide solutions contained 0.01 M sodium acetate and 0.02 M NaCl unless stated otherwise. The pH was adjusted to 5.8 with 0.1 M HCl. The ribonuclease A concentrations were measured on a Cary 16 spectrophotometer, using an absorptivity value of 0.71 L/(cm g) at 277 nm (Harrington and Schellman, 1956). *N*-Acetyl-L-tyrosinamide concentrations were measured by taking the optical density at 274 nm where the molar extinction coefficient is 1400 (Sober and Harte, 1968). The same value of the ribonuclease A extinction coefficient was used at all solvent compositions, since it has been shown that, at pH 5.8, variation of solvent composition from water to 50% MPD does not affect seriously the position of the absorption maximum nor the extinction coefficient of this protein (Pitts and Bello, 1973).

Refractive Index Increments. Refractive index increments of RNase A were measured on a photoelectric differential refractometer at 436 nm and 25 °C (Pittz and Bablouzian, 1973). For measurements of the refractive index increment at identical chemical potentials of solvent components in the protein solution and the reference solvent, $(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$, the ribonuclease A solution was first brought to dialysis equilibrium with the solvent; the dialyzed protein and dialysates were then introduced into the two compartments of the differential cell. For measurements of the refractive index increment at conditions at which solvent component molality was kept identical in the protein solution and reference solvent, $(\partial n/\partial C_2)_{T,p,m_3}$, the protein was dried at 45 °C under a vacuum of less than 0.05 μm for 18 h, and the sample solution and reference solvent were then prepared by weighing out identical amounts of water and MPD into each. The initial protein concentration was 5 mg/mL. After measurement of the differential refractive index at the highest protein concentration, the stock protein solution was diluted gravimetrically with solvent and the measurement performed again. In this way, measurements were taken at a series of protein concentrations down to 0.75 mg/mL. These values of dn/dC_2 obtained were found to be independent of protein concentration.

The refractive index increments of MPD in aqueous solutions, $(\partial n/\partial C_3)_{T,P,m_2}$, were obtained by measuring the refractive index at several solvent compositions with a Bausch and Lomb precision refractometer at 436 nm and 25 °C and drawing tangents to the plot of the refractive index vs. the concentration of MPD.

Light-scattering measurements were carried out on a Brice-Phoenix photometer which had been adapted to a digital readout (Timasheff and Townend, 1970; Pittz et al., 1975). A stock solution of RNase was prepared by matching solvent compositions in both sample and solvent in a manner similar to that used for the refractive index increment measurements. The solutions were then clarified for light scattering by centrifugation in a Beckman Model L265B centrifuge at 40 000 rpm for 30 min in a 65 rotor, followed by filtration through an ultrafine sintered glass filter of special design (Bier, 1957).

In carrying out the light-scattering experiments, a clean Dintzis type cell (Timasheff et al., 1957) was tared and approximately 2.1 mL of the appropriate H_2O–MPD solvent was added after filtration through an Ultrafine sintered-glass filter. The cell was then weighed and the light scattering of the solvent determined. Increments of the ribonuclease A stock solution in exactly the same H_2O–MPD solvent was then added from an ultramicroburette, the cell being weighed after each addition and checked for evaporation and leaks by weighing as a function of time. After each addition, the intensity of the light scattered was measured in the usual way. Although the stock solutions containing ribonuclease A were added with ultramicroburettes, the concentrations were determined by weight. The mixing procedure which consisted in inversion and rocking of the Teflon-stoppered cell caused bubble formation in this solvent system. It was found in general that in a viscous solvent, such as 50% aqueous MPD, several hours were required for the solution to clear of bubbles. Additions of protein stock solution to the cell were repeated until the protein concentration had reached 2 to 5 mg/mL. The concentration of the protein in the cell (C_n) for each light-scattering point was then calculated by[2]

$$C_n = \frac{g_n}{g_n + g_{solv}} C_t \qquad (1)$$

where g_n is the weight of protein stock solution added; g_{solv} is the weight of solvent; C_t is the concentration of the stock solution of protein in g per mL.

For experiments with dialysis, at least ten solutions of ribonuclease A at different concentrations were prepared in a given H_2O–MPD mixture, dialyzed for 7 to 10 days against a large excess of the same solvent and passed through the sintered glass filter after centrifugation. The light-scattering measurements were then carried out in Dintzis type cells, using the dialysate as blank. Concentrations of the dialyzed RNase A solutions were measured spectrophotometrically after a fivefold dilution with buffer.

Circular Dichroism Measurements. Circular dichroism (CD) measurements were performed at 23 °C on a Cary Model 60 spectropolarimeter with a 6001 CD attachment with slit width programmed for a band resolution of 1.5 nm. The time constant was either 3 or 10 s. Mean residue ellipticities, $[\theta]_{MRW}$, reported in deg cm^2/dmol residue, were calculated using a mean residue weight of 115 for ribonuclease A and 223 for *N*-acetyl-L-tyrosinamide. The ribonuclease A concentrations were 1.0 to 1.5 mg/mL. The path length was 10 mm in the near ultraviolet and 0.1 mm in the far ultraviolet.

The partial specific volumes of MPD at various solvent compositions were obtained from pycnometric density measurements and the graphical method of intercepts (Lewis and Randall, 1923). The results are presented in Table I.

[2] The expression is not exact since the densities of the protein solutions and of the solvents are not identical. The resulting errors are, however, insignificant.

Theory. Preferential interactions between proteins and solvent components in mixed solvents may be determined by a variety of thermodynamic techniques, such as light scattering (Ewart et al., 1946; Kay and Edsall, 1956; Read, 1960; Stauff and Mehrotra, 1961; Inoue and Timasheff, 1968a,b; Timasheff and Inoue, 1968; Pittz et al., 1975), differential refractometry (Vrij, 1959; Casassa and Eisenberg, 1961; Vrij and Overbeek, 1962; Noelken and Timasheff, 1967; Inoue and Timasheff, 1968a,b; Timasheff and Inoue, 1968; Pittz et al., 1975), sedimentation equilibrium (Schachman and Edelstein, 1966), densimetry (Kielley and Harrington, 1960; Casassa and Eisenberg, 1964; Cohen and Eisenberg, 1968; Reisler et al., 1977; Lee and Timasheff, 1974), small-angle x-ray scattering (Luzzati et al., 1961, 1967; Timasheff, 1973; Pessen et al., 1973), vapor pressure equilibrium (Hade and Tanford, 1967; Bull and Breeze, 1970a,b), equilibrium dialysis and gravimetric measurements (Bull and Breeze, 1970a,b), etc. The various methods which may be used all result in a preferential interaction, or preferential "binding" parameter (Casassa and Eisenberg, 1964; Tombs and Peacocke, 1974)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \frac{M_2}{M_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} \tag{2}$$

where g_i is the concentration of component i in grams of i per gram of component 1, in the present case water, m_i is the molal concentration of component i (moles of component i per 1000 g of principal solvent), M_i is the molecular weight of component i, T is the thermodynamic temperature, and μ_i is the chemical potential of component i. Using the notation of Scatchard (1946) and Stockmayer (1950), component 1 is the principal solvent, in this case water, component 2 is the protein and component 3 is the added solvent component, in the present case MPD. Depending on whether component 3 interacts preferentially with component 2, or is preferentially excluded from its domain, the interaction parameter will take on positive or negative values. A negative value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ indicates a deficiency of component 3, i.e., preferential interaction of the protein with component 1 (when the principal solvent is water, this is preferential hydration) (Timasheff and Kronman, 1959)

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = -\frac{1}{g_3}\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} \tag{3}$$

The degree of preferential interaction, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is defined as zero when the number of grams of third component per gram of water is identical on both sides of the membrane at osmotic equilibrium. On the other hand, it is also possible to define as zero preferential interaction the state in which the amount of third component per mL of solution is identical on the two sides of the dialysis membrane $(\partial C_3/\partial C_2)_{T,\mu_1,\mu_3}$, where C_i is the concentration of component i in g per mL of solution. This definition does not reflect correctly the thermodynamic interaction, since it does not consider the volume occupied by the protein and includes, as part of the interaction, dilution of the solvent by the addition of the protein (Inoue and Timasheff, 1968a,b). The two types of measurements are related by (Noelken and Timasheff, 1967)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \frac{1}{(1-\bar{v}_3 C_3)}\left(\frac{\partial C_3}{\partial C_2}\right)_{T,\mu_1,\mu_3} \tag{4}$$

where $\bar{v}_3$ is the partial specific volume of the solvent.

At this point, it seems desirable to examine the exact physical nature of preferential interaction. This quantity refers to the excess of a solvent component relative to the bulk mixed solvent which is present as a time average in the immediate domain of the macromolecule. It is frequently referred to as preferential binding. Yet, it in no way implies the formation of stoichiometric complexes between the macromolecule and solvent components, nor any strong binding, as would be involved in the formation of "iceberg" or other structures nor indeed contact between the interacting molecules. Preferential interaction is a pure thermodynamic quantity; it is simply a reflection of the perturbation of the chemical potential or activity coefficient of component 3 by addition of the macromolecule, since

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\frac{\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3}}{\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}} \tag{5a}$$

$$\mu_i = \mu_i^\circ + RT \ln a_i = \mu_i^\circ + RT \ln m_i \gamma_i$$
$$= \mu_i^\circ + RT \ln m_i + \mu_i^{(e)} \tag{5b}$$

where a_i is the activity of component i, γ_i is its activity coefficient and $\mu_i^{(e)}$ is the excess chemical potential and is equal to $RT \ln \gamma_i$. At chemical equilibrium, the chemical potentials, and hence, activities, of solvent components must be identical throughout the system, namely, in the immediate vicinity of the macromolecule and at infinite distance in the bulk solvent. Since, unless $(\partial \mu_3/\partial m_2)_{T,P,m_3}$ is zero, introduction of the macromolecule into the system causes a change in the activity coefficients of solvent components in its domain, the concentrations of the solvent components in this domain must also change. As a result, preferential interaction can be expressed in terms of a net "binding" of solvent molecules to the macromolecule, although the actual interactions involved may span the energy spectrum from strong complex formation at specific sites to a momentary perturbation of the freedom of rotation or translation of a solvent molecule by attraction or repulsion by the macromolecule. It is evident, therefore, that it is meaningless to speak of numbers of molecular layers of solvent component molecules immobilized around the macromolecule when discussing preferential interactions. Preferential interaction, expressed as "binding", is related to total interactions between the macromolecule and solvent components by (Timasheff, 1963; Inoue and Timasheff, 1972; Kupke, 1973; Reisler et al., 1977)

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = A_3 - g_3 A_1 \tag{6}$$

where A_i is the total amount of compound i "bound" to component 2, expressed in grams of component i per gram of component 2. Examination of eq 6 reveals that the sign of the preferential interaction parameter may vary, depending on solvent composition, g_3, and the amounts of solvent components interacting with the protein, A_1 and A_3. It is possible to have a situation in which the degree of interaction between a solvent component and protein increases monotonely as the bulk solvent composition is enriched with respect to that component, while the preferential interaction displays a more complicated pattern (Timasheff, 1970). In fact, in the case of the interaction of various proteins with solvent components in the water–2-chloroethanol solvent system, it has been found that $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ first increases, then, after passing through a maximum, it decreases, and, at high 2-chloroethanol concentrations, it becomes negative, even though the total interaction of 2-chloroethanol with the proteins increases monotonely (Inoue and Timasheff, 1968a,b; Timasheff and Inoue, 1968; Timasheff, 1970).

In the present study, methods chosen for measuring preferential interactions were differential refractometry and light

TABLE II: Preferential Interactions of Ribonuclease A with Solvent Components in the Water-2-Methyl-2,4-pentanediol System at pH 5.8.

% MPD (mL/ml of solution)	$\left(\frac{\partial n}{\partial C_2}\right)_{T,\mu_1,\mu_3}$ (mL/g)	$\left(\frac{\partial n}{\partial C_2}\right)_{T,P,m_3}$ (mL/g)	$\left(\frac{\partial n}{\partial C_3}\right)_{T,P,m_2}$ (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial\mu_3/\partial m_2)_{T,P,m_3}$ [cal/(mol in 1000 g of H_2O)²]
20	0.170 ± 0.002	0.175 ± 0.003	0.136	−0.045 ± 0.045	0.196 ± 0.106	150	1600
30	0.152 ± 0.003	0.171 ± 0.002	0.122	−0.215 ± 0.057	0.555 ± 0.146	425	4500
40	0.130 ± 0.001	0.162 ± 0.001	0.107	−0.474 ± 0.031	0.810 ± 0.051	620	6500
50	0.103 ± 0.002	0.156 ± 0.001	0.109	−0.943 ± 0.053	1.031 ± 0.058	790	8700

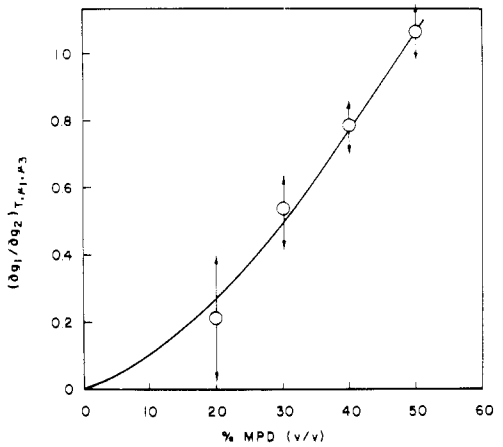

FIGURE 1: Preferential interaction of ribonuclease A with solvent components in the water–MPD system at pH 5.8, 25 °C.

scattering, because of the large difference between the refractive indices of water and MPD, 1.340 and 1.435, respectively, at 25 °C.

Refractive index is an intensive property and its change with variation in solvent composition is given by

$$dn = \left(\frac{\partial n}{\partial g_2}\right)_{T,P,g_3} dg_2 + \left(\frac{\partial n}{\partial g_3}\right)_{T,P,g_2} dg_3 \qquad (7)$$

where n is the refractive index of the solution.

Taking the partial derivative of eq 7 with respect to protein concentration at constant chemical potential of component 3 results in the working equation for calculating preferential interactions from refractive index gradients:

$$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,P,\mu_3} = \frac{(\partial n/\partial g_2)^\circ_{T,P,\mu_3} - (\partial n/\partial g_2)^\circ_{T,P,m_3}}{(\partial n/\partial g_3)_{T,P,m_2}} = \frac{1}{(1 - C_3\bar{v}_3)}\left[\frac{(\partial n/\partial C_2)^\circ_{T,P,\mu_3} - (\partial n/\partial C_2)^\circ_{T,P,m_3}}{(\partial n/\partial C_3)_{T,P,m_2}}\right] \qquad (8)$$

The superscript ° indicates extrapolation to zero protein concentration. The term $(\partial n/\partial C_2)_{T,P,\mu_3}$ is equal within experimental error to $(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$, i.e., to the increment measured at dialysis equilibrium at atmospheric pressure (Stigter, 1960). This eliminates the necessity of carrying out the operations under a hydrostatic head equal to the osmotic pressure of the solution.

In the case of light scattering, when the measurements are done keeping solvent component molalities identical in the protein solution and the reference solvent, multicomponent theory results in the equation (Zernicke, 1918; Brinkman and Hermans, 1949; Kirkwood and Goldberg, 1950; Stockmayer, 1950; Timasheff et al., 1957; Timasheff and Kronman, 1959; Casassa and Eisenberg, 1960, 1961, 1964; Stigter, 1960, 1963; Inoue and Timasheff, 1968b).

$$H\frac{C_2}{\Delta\tau} = \frac{32\pi^3 n^2}{3N\lambda^4}(\partial n/\partial C_2)^2_{T,p_1,m_3}\frac{C_2}{\Delta\tau} = \frac{1}{(1+D)^2}\left[\frac{1}{M_2} + 2B^\circ C_2 + 0(C_2^2)\right] \qquad (9a)$$

$$D = \frac{(\partial n/\partial m_3)_{T,p,m_2}}{(\partial n/\partial m_2)_{T,p,m_3}}(\partial m_3/\partial m_2)_{T,p,\mu_3} = \frac{(1 - C_3\bar{v}_3)_{m_2}}{(1 - C_2\bar{v}_2)m_3}\frac{(\partial n/\partial C_3)_{T,p,m_2}}{(\partial n/\partial C_2)_{T,p,m_3}}(\partial g_3/\partial g_2)_{T,p,\mu_3} \qquad (9b)$$

In these equations, $\Delta\tau$ is the difference between the turbidity of the solution and that of the solvent, N is Avogadro's number, λ is the wavelength of the light in vacuo, and B° is the second virial coefficient (Inoue and Timasheff, 1968a,b). The partial specific volumes in eq 9b are measured at equal molalities of third component in the solution and reference solvent.

The true molecular weight of the macromolecule can be measured by light scattering (Stigter, 1960, 1963; Casassa and Eisenberg, 1960, 1964), if all the measurements are made at conditions at which the chemical potentials of the solvent components are kept identical in the solution and in the solvent, i.e., after dialysis equilibrium. Under such conditions, the three component equation reduces to the pseudo-two-component form

$$\frac{32\pi^3 n^2}{3N\lambda^4}(\partial n/\partial C_2)_{T,p,\mu_3}\frac{C_2}{\Delta\tau} = \frac{1}{M_2} + 2B'C_2 \qquad (10)$$

This makes it possible to determine the true molecular weight of the protein, M_2, and the degree of molecular association in a three-component system. The difference between the reciprocals of the intercepts of eq 9a and 10 gives the term D, from which the preferential interaction between components 3 and 2 can be readily calculated with eq 9b.

Results

The results of differential refractometry experiments on the system ribonuclease A–water–MPD at pH 5.8 are presented in Table II as a function of solvent composition. The values of column 5 show that, at all solvent compositions, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is negative, indicating a deficiency of MPD in the domain of the protein. The corresponding values of preferential hydration, calculated with eq 2, are shown in columns 6 and 7 of this table and in Figure 1. It is seen that the interaction is marked by strong preferential hydration of the protein, the extent of this interaction increasing in monotone fashion with increase in the solvent contents of MPD and reaching a value of 1 g of water per g of protein. These results are in qualitative agreement with those of Pittz and Bello (1971) who reported that cross-linked ribonuclease A crystals were preferentially hydrated to the extent of 0.3–0.4 g of water per g of protein. A direct comparison of the values is not feasible, since it is very difficult to establish the thermodynamic standard state of their experiments. Furthermore, it is reasonable to expect that the

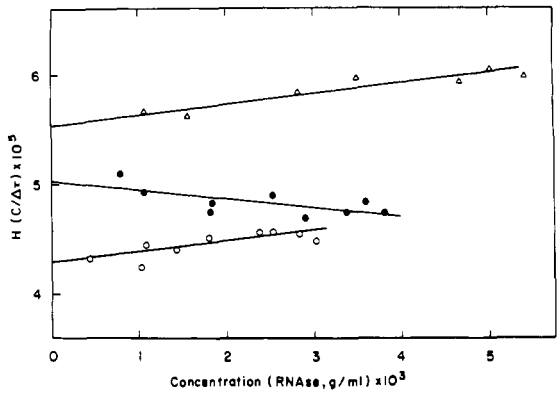

FIGURE 2: Light scattering of ribonuclease A in the water–MPD system at 25 °C. The solvents are: (△) 0.02 M NaCl, 0.01 M sodium acetate buffer, pH 5.8; (●) same solvent, with 30% (v/v) MPD, at constant m_3; (○) same solvent, with 30% (v/v) MPD, at constant μ_3.

TABLE III: Light Scattering of Ribonuclease A in H_2O–2-Methyl-2,4-pentanediol, pH 5.8.

MPD (vol %)	M_2(app)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)
0	18 100		
30 (const μ_3)	23 300		
30 (const m_3)	20 000	−0.15 ± 0.05	0.38 ± 0.16
50 (const μ_3)	24 500		
50 (const m_3)	9 800	−1.07 ± 0.20	1.21 ± 0.23

degree of interaction with solvents would be considerably reduced in cross-linked crystals by the close packing of the protein molecules.

The last column of Table II shows the change in the chemical potential of MPD induced by introduction of ribonuclease A into the solvent. This was calculated from the data of column 5 with eq 5a. In this calculation

$$(\partial\mu_3/\partial m_3)_{T,p,m_2} = (RT/m_3) + RT(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$$

was approximated by RT/m_3, since no data are available on the variation of the activity coefficient of MPD with concentration. The error introduced is, however, not large and does not affect the values of $(\partial\mu_3/\partial m_2)_{T,P,m_3}$ by more than 10%. At all solvent compositions, introduction of protein into the water–MPD system causes the chemical potential of MPD to become more positive. This indicates repulsion between ribonuclease and MPD and, hence, a thermodynamic destabilization of the system which increases with an increase in MPD concentration.

The results of the light-scattering experiments are shown in Figure 2 and Table III. Again, the interaction parameters indicate strong preferential hydration. Some aggregation of the protein is also evident. In dilute buffer, the weight-average molecular weight is found to be 18 100, while the molecular weight of the monomeric enzyme is known to be 13 700. This is in agreement with previous reports on the state of aggregation of ribonuclease in aqueous solution (Hirs et al., 1953; Van Holde and Baldwin, 1958; Yphantis, 1964). Indeed, Yphantis (1964) found a weight average molecular weight of 17 500 for ribonuclease A in dilute phosphate buffer at pH 7.8. Addition of MPD causes further aggregation, the weight-average molecular weight increasing to 23 300 and 24 500 in 30% and 50% MPD, respectively. Comparison of the values of preferential hydration obtained by light scattering with those measured by differential refractometry shows the two to be in general

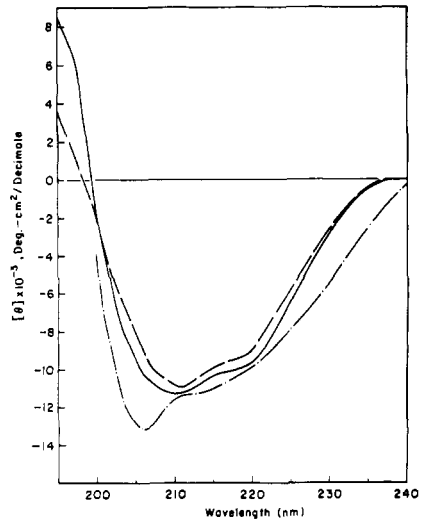

FIGURE 3: Far-UV CD spectra of ribonuclease A at pH 5.8. (—) with 0.02 M NaCl, 0.01 M sodium acetate buffer; (- -) same solvent, with 50% (v/v) MPD; (- · -) same solvent, with 50% (v/v) 2-chloroethanol.

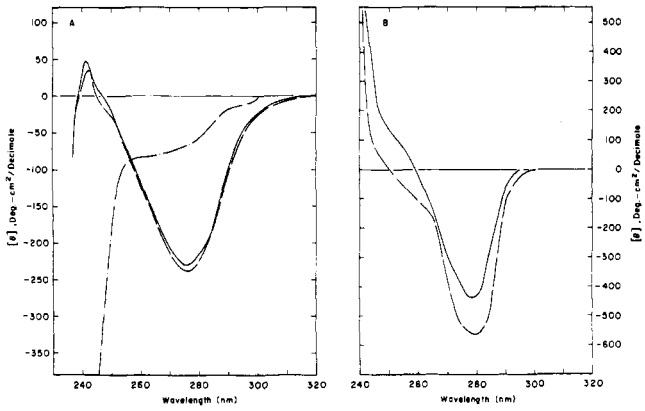

FIGURE 4: Near-UV CD spectra. (A) Ribonuclease A at pH 5.8: (—) 0.02 M NaCl, 0.01 M sodium acetate buffer; (- -) same solvent, with 50% (v/v) MPD; (- · -) same solvent, with 50% (v/v) 2-chloroethanol. (B) *N*-Acetyl-L-tyrosinamide: (—) 0.02 M NaCl, 0.01 M sodium acetate, pH 5.8, buffer; (- -) same solvent with 50% (v/v) MPD.

agreement, although the aggregation of ribonuclease under the conditions of these experiments leads to rather large uncertainties in the values of D in eq 9.

The circular dichroism spectra of RNase A at pH 5.8, 0.01 M sodium acetate, 0.02 M NaCl, were obtained at 0, 10, 20, 30, 40, and 50% MPD and 50% 2-chloroethanol. The spectra in 50% MPD, 50% 2-chloroethanol and in dilute buffer are shown in Figures 3 and 4a. In the far-ultraviolet region, the difference between the spectra in dilute buffer and 50% MPD is within experimental error, in agreement with Pittz and Bello (1971), while the spectrum of RNase in 2-chloroethanol differs markedly from that in dilute buffer. The negative maximum at 211 nm, with an ellipticity of −11 300 deg cm^2/dmol is in agreement with literature values (Simpson and Vallee, 1966; Timasheff et al., 1967). In the near ultraviolet region, shown in Figure 4a, the spectra in 50% MPD and dilute buffer are again almost identical, while that in 50% 2-chloroethanol indicates major changes in conformation. These results indicate that transfer of ribonuclease A from dilute buffer to 50% MPD affects neither the secondary structure of the protein nor the asymmetry of the environment and degree of rotational constraint of the tyrosine residues and disulfide bridges which give rise to the near-UV bands. On the other hand, transfer to 2-

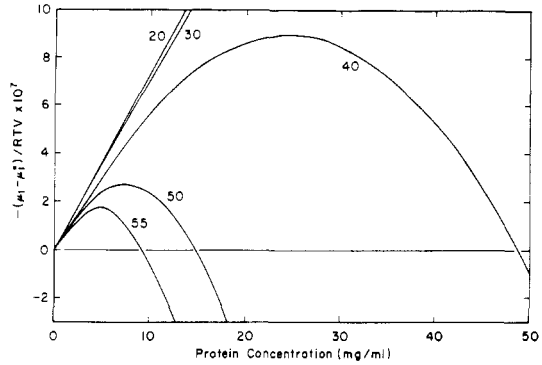

FIGURE 5: Effect of MPD on the chemical potential of water as a function of protein concentration, at pH 5.8, 25 °C. The numbers on the curves refer to the volume percent of MPD present in the system.

chloroethanol, a solvent of similar free energy of transfer per residue, leads to a major structural rearrangement.

Since a change in environment from aqueous to 50% MPD can be expected to perturb the CD spectra of exposed aromatic residues, near-ultraviolet spectra were obtained in the two media for the model compound *N*-acetyl-L-tyrosinamide. The results shown in Figure 4b indicate a definite perturbation of the tyrosine chromophore spectrum by MPD. There is both an increase in intensity of the negative band and a red shift (by ca. 3 nm) of the maximum position, as may be expected when the dielectric constant of the environment decreases. The essential lack of such a shift in the same bands of ribonuclease A suggests that the dielectric constant of the tyrosine ring environment in the enzyme is not significantly affected by a change of medium from aqueous to 50% MPD. This can be due to either the fact that exposed tyrosine residues contribute little to the near-UV CD spectrum of ribonuclease A or to an essential lack of contact of surface tyrosines with MPD molecules, as a result of the strong preferential hydration. In either case, the near-UV spectral results support the conclusion that MPD does not significantly affect the tertiary structure of the macromolecule.

Discussion

The present experiments have led to three principal observations: First, dispersion of ribonuclease A in 50% MPD results in a thermodynamic destabilization of the solvent system, manifested through an increase in the chemical potential of MPD and a strong preferential hydration of the protein; second, the secondary and tertiary structures of the protein are not perturbed within the criteria of detectability by circular dichroism; third, the aggregation of ribonuclease is enhanced. Let us demonstrate that the three effects are related, and that the first two observations can account for the ability of this solvent system to induce the crystallization of ribonuclease A.

A thermodynamic destabilization of the system, as manifested by a positive increment in the chemical potential of MPD when ribonuclease A is introduced into the water–MPD mixture, will tend to drive the system to a new state of equilibrium. In a protein system, this may occur either through a change in the conformation of the protein to a state in which it can form thermodynamically favorable contacts with the cosolvent, or through a reduction in contacts between protein and cosolvent. The latter may be achieved either by phase separation (crystallization of precipitation) or by protein aggregation, which, in fact, may be regarded as a micro phase separation. In other words, the system may change either to a state in which the cosolvent changes its character from precipitant to good solvent or to one in which the macromolecules are removed from contact with the poor solvent.

In the case of ribonuclease A, no conformational change occurs in 50% MPD. Therefore, phase separation may be expected. For a three-component system, the equation of state may be written as (Tanford, 1961; Casassa and Eisenberg, 1964):

$$\frac{\mu_1 - \mu_1^\circ}{RTV_1} = -\frac{C_2}{M_2}\left\{1 + \frac{V_m}{2RTM_2} C_2[(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3} + (\partial\mu_2/\partial m_3)_{T,P,m_2}(\partial m_3/\partial m_2)_{T,P,\mu_3}] + 0(C_2^2)\right\} \quad (11)$$

where μ_1 is the chemical potential of the principal solvent (water), V_1 is its molar volume, and V_m is the volume of solution which contains 1000 g of principal solvent.[3] Negative values of $(\mu_1 - \mu_1^\circ)$ indicate a stable solution system, a value of zero corresponds to a θ solvent, positive values indicate instability of the system and, consequently, phase separation (Flory, 1953; Nord et al., 1951).

Using the values of $(\partial\mu_2/\partial m_3)_{T,P,m_2} = (\partial\mu_3/\partial m_2)_{T,P,m_3}$ and $(\partial m_3/\partial m_2)_{T,P,\mu_3} \simeq (\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ of Table II, setting the self-interaction term, $(\partial\mu_2^{(e)}/\partial m_2)_{T,P,m_3}$ equal to the excluded volume effect (Tanford, 1961) and neglecting terms in higher powers of protein concentration, the chemical potential of water as a function of ribonuclease A concentration was calculated for the water–MPD systems, containing 20, 30, 40, and 50% MPD. The results are shown in Figure 5, as a series of isotherms. In 40 and 50% MPD, the chemical potential of water is seen to assume first negative values, then, after passing through a maximum, to become less negative and, finally, to become positive after crossing zero at a given protein concentration. The cross-over points occur at 49 and 15 mg/mL ribonuclease A in solvents containing 40 and 50% MPD, respectively, while the minima are at 25 and 8 mg/mL concentrations. One can expect, therefore, that, in these solvents, phase separation will occur and protein will either precipitate or crystallize at concentrations above these values. It is noteworthy that ribonuclease A has been crystallized out of 50% aqueous MPD at total protein concentrations of 25 mg/mL (King et al., 1956). In 30% MPD the cross-over point is calculated to occur at a protein concentration of ca. 700 mg/mL.

[3] This equation follows from the combination of the thermodynamic definition of osmotic pressure, π

$$\mu_1 - \mu_1^\circ = -\pi V_1 \quad (11a)$$

with the virial expansion given by Casassa and Eisenberg (1964)

$$\frac{\pi}{RT} = \frac{1}{V_m}(m_2 + B^{(w)}M_2^2 + \ldots) \quad (11b)$$

where

$$B^{(w)} = \frac{1}{2}\left(a_{22} - \frac{a_{23}^2}{a_{33}} - \frac{1}{m_2}\right) \quad (11c)$$

and

$$a_{ij} = \left(\frac{\partial \ln a_i}{\partial m_j}\right)_{T,P,m_{k\neq j}} = \left(\frac{\partial \ln a_j}{\partial m_i}\right)_{T,P,m_{k\neq i}} \quad (11d)$$

and a_i is the molal activity of component i. Equations 11b and 11c are reduced forms of the complete osmotic pressure equation. Casassa and Eisenberg (1964) have given criteria for testing the applicability of these simplified equations for any given system. Application of these criteria to the water–MPD system has shown that eq 11b and 11c are valid for this system within a close approximation. Furthermore, Ross and Minton (1977) have recently discussed the role of higher virial terms in concentrated protein solutions and have shown that these became significant at concentrations considerably higher than those used in the present study.

In 20% MPD, $(\mu_1 - \mu_1^\circ)$ remains negative at all protein concentrations.

Since for the x-ray crystallographic structural studies, ribonuclease A was crystallized from 55% MPD (Kartha et al., 1967), a calculation was carried out for this solvent composition as well, although no interaction data are available. The necessary parameters were estimated by extrapolating the curve of Figure 1 to 55% MPD. The values obtained were $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = -91$ and $(\partial \mu_3/\partial m_2)_{T,P,m_3} = 9300$. Substitution of these values into eq 11 gave the results shown in Figure 5. These indicate that phase separation should occur above a concentration of 5 mg/mL. Again, King et al. (1956) used 25 mg/mL protein solutions in the preparation of their crystals for x-ray crystallography.

The same concepts can be used to explain the observed enhancement of ribonuclease A aggregation upon addition of MPD. It is known (Wyman, 1964; Tanford, 1969; Aune et al., 1971; Lee and Timasheff, 1977) that for any reaction which depends on a solvent variable, at constant temperature, pressure, and activity of all other solution components, the equilibrium constant, K, will vary with solvent composition as

$$\frac{d \ln K}{d \ln a_x} = \Delta\bar{\nu}_x \qquad (12)$$

where a_x is the activity of solvent component x and $\Delta\bar{\nu}_x$ is the difference between the preferential interactions of macromolecule and component x in the final and initial states of the reaction. Using the notation of this paper for a system aggregating to the extent n

$$\Delta\bar{\nu}_x = \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}^{(\text{aggregate})} - n\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}^{(\text{monomer})} \qquad (13)$$

In the case of ribonuclease A in the water–MPD system, the preferential interaction is negative. This interaction is nonspecific, being preferential exclusion of MPD from contact with protein. Therefore, as an average, it acts uniformly over the entire surface of the protein. Since the protein conformation is the same in dilute buffer and in the water–MPD system, its surface area may be regarded within close approximation as identical in the two media. Aggregation causes the removal of part of this surface from contact with solvent by formation of protein–protein contact, reducing the absolute value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ per monomeric subunit when the protein aggregates. This quantity being negative, $\Delta\bar{\nu}_x$ should be positive and the association constant should increase, with a resultant higher extent of aggregation. In the case of ribonuclease A, the known aggregation is enhanced by changing the medium to water–MPD mixtures. It should be noted that eq 12 and 13 are rigorously valid only for systems in equilibrium and cannot be applied directly to the observed aggregation of ribonuclease. Nevertheless, their use gives a qualitative rationale for the enhancement of ribonuclease aggregation by addition of MPD to the system.

Having established that the crystallization of ribonuclease A out of 50% MPD is the result of the unfavorable interaction between MPD and the protein, it seemed interesting to examine the reasons for this unfavorable effect. Taking the results of Table II, if it is assumed that MPD is totally excluded from the domain of the protein, i.e., if one sets $A_3 = 0$ in eq 6, the minimum interaction with water is given by the values of column 6 of Table II. Thus, in 50% MPD, there is an effective layer of water of 1 g per g protein which is impenetrable to MPD. This is three times the value of the normal hydrodynamic hydration of proteins (Oncley, 1943; Edsall, 1953) or of hydration measured by vapor pressure (Bull and Breeze, 1970a,b; Hade and Tanford, 1967), or NMR techniques (Kuntz, 1971a,b: Kuntz and Kauzman, 1974). It appears, therefore, that, close to the ribonuclease molecule, there is a considerable region in which solvent composition is perturbed. Since this cannot be accounted for in terms of the usual hydration of the protein, for example, of the charged residues, and since, in effect, it is the repulsion of one solvent component from the domain of the protein, rather than strong attraction of the other which drives this reaction, it must be concluded that the mechanism of this interaction is through an effect of the protein on the structure of the solvent, rather than that of the solvent on the structure of the protein.

TABLE IV: Mole Fraction[a] of Salt Needed to Salt Out Cosolvents at 25 °C.

Salt	50% MPD	50% chloroethanol
NaCl	2.65×10^{-2}	No separation
Na acetate	2.95×10^{-2}	No separation
Na_2SO_4	0.33×10^{-2}	0.58×10^{-2}
Na_3 citrate	0.29×10^{-2}	0.60×10^{-2}
KCl	2.54×10^{-2}	No separation
K acetate	2.61×10^{-2}	No separation
$(NH_4)_2SO_4$	0.64×10^{-2}	1.53×10^{-2}
Guanidine SO_4	2.36×10^{-2}	8.45×10^{-2}
$(CH_3)_4NBr$	1.1×10^{-2}	No separation
$CaCl_2$	2.43×10^{-2}	No separation
Mg $acetate_2$	2.88×10^{-2}	No separation
$MgSO_4$	0.23×10^{-2}	0.54×10^{-2}

[a] Calculated on the basis of formula weight for the salts.

This conclusion is totally at variance with what had been reported previously for several proteins at low pH in mixed solvents in which the nonaqueous component was 2-chloroethanol, methoxyethanol, or ethylene glycol (Inoue and Timasheff, 1968a,b; Timasheff and Inoue, 1968; Timasheff, 1970). In all of these solvent systems, the principal effect was one of direct interaction of the organic solvent component with the protein accompanied by a gradual unfolding of the protein, the extent of which was proportional to the amount of the organic solvent component interacting with the protein.

The free energy of transfer of amino acid side chains from water to 50% MPD being not very different from that from water to denaturing alcohols (Pittz and Bello, 1971), the failure of the protein to unfold in this medium must be a consequence of the exclusion of MPD from the protein surface and the resulting low probability of it forming direct contacts with the nonpolar residues of the protein. On the other hand, 50% 2-chloroethanol does induce a strong conformational change in ribonuclease A at neutral pH as evidenced by the CD spectra shown in Figures 3 and 4. In the near UV the 277-nm band is abolished, only the residual rotation due to disulfide transitions remaining. In the far-UV region the intensity is increased and the negative maximum shifts to lower wavelengths with the generation of a spectrum typical of denatured proteins (Fasman et al., 1970). The causes of this difference between the ability of these various solvents to come into contact with the protein surface must be looked for in intrinsic differences between these solvent systems, in the thermodynamic stability of their interactions with water and, in particular, in differences on the micro scale in their ability to approach points of different chemical characters on the surface of the protein and to compete with water molecules for such interactions. In relation to this, we have observed that low concentrations of neutral salts are able to induce phase separation in the water–MPD system, at neutral pH. Some typical results are presented in Table IV. These show that 50% MPD solutions become unstable at salt formula weight mole fractions between 2×10^{-3} and 3×10^{-2}.

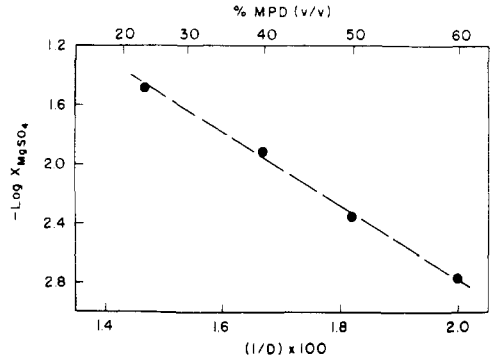

FIGURE 6: Effect of $MgSO_4$ on the thermodynamic stability of water–MPD systems. For details, see text.

Similar data for 50% 2-chloroethanol show a much higher stability of this solvent system in the presence of salts. In fact, in this system, phase separation frequently did not occur right up to saturation with the salt. Ethylene glycol in a 50% mixture with water was not salted out by any of these salts. It is evident from these results, that water–MPD solutions are highly destabilized by contact with charged particles which cause phase separation, the salt remaining in the aqueous phase.

The phase separation as a function of MPD concentration was examined in $MgSO_4$, since this had been found to be the strongest salting out agent. The results, shown in Figure 6, indicate compliance with the standard salting out relation: $-\ln x_{salt} = a + b/D$, where x_{salt} is the mole fraction of salt at phase separation, D is the dielectric constant of the solution, and a and b are constants (Edsall and Wyman, 1958).

If one now considers on the micro scale the interactions at the protein–solvent interface, the protein surface may be regarded as being covered by a mosaic of charges which can interact individually with solvent molecules. By extrapolating to proteins the effects of dilute salts on the solvent system, it can be expected that a micro phase separation of the water–MPD system will occur about each charged point. Since the charges are fixed in space to the protein molecule, they cannot migrate into a separate aqueous phase. Therefore, it is the water molecules which must migrate to the protein surface, while MPD molecules must be excluded from it. Thermodynamically, such an effect would be reflected by a sharp increase in the activity coefficient of MPD upon contact with the protein and preferential hydration of the latter. It is this inability of MPD molecules to come into contact with ribonuclease which prevents denaturation of the protein. To the contrary, 2-chloroethanol, which is much less affected by salts can penetrate to the protein surface and cause its denaturation.

The proposed mechanism for the strong preferential hydration of ribonuclease A in MPD at pH 5.8 is supported by an additional observation, namely, in 0.01 M HCl, where the total number of charges on the enzyme is considerably smaller than at neutral pH, the preferential hydration of ribonuclease A in 50% aqueous MPD is smaller than at neutral pH and the protein undergoes a partial unfolding.[4]

From the above considerations, it seems reasonable to conclude that the crystallization of ribonuclease A out of water–MPD mixtures is caused by the "salting out" of MPD by the charges on the protein surface. This unfavorable thermodynamic interaction leads to phase separation, i.e., crystallization. The accompanying strong preferential hydration of the protein hinders MPD molecules from coming into contact with the protein surface in a concentration sufficient to interact significantly with the nonpolar residues and to induce, in this manner, conformational changes. As a result, the protein is crystallized in its native structure.

[4] Pittz and Timasheff, to be published.

Dedication

This paper is dedicated to the memory of the late Professor Jerome Vinograd.

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