Effects of the Solvent Medium on Polyvalylribonuclease Aggregation*

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ABSTRACT: The aggregation of polyvalylribonuclease A (PVRNase) has been studied as a model system which exhibits nonpolar as well as electrostatic interactions like those involved in the denaturation and subunit association of proteins. Aggregation was followed turbidimetrically at 320 mμ at 39°. The enzymatically active derivative aggregated between pH 6 and pH 8.2 at 0.38 mg of protein/ml. The sodium salts of sulfate, phosphate, and pyrophosphate shifted the pH–aggregation rate profile in an order similar to that observed for the multivalent anion protection of native RNase against urea denaturation. The lowering of PVRNase aggregation was seen at low (below 0.05 M) concentrations of anions. The aggregation rate was found to change sigmoidally with increase in ionic strength. At salt concentrations up to about 0.15 M, the log of the relative rate increased linearly with the square root of the ionic strength. At high concentrations of salt (above 0.2 M) the aggregation rate increased (salting out) or decreased (salting in) depending on the type of salt. Nonelectrolytes diminished the aggregation at high concentrations of solute. The data presented indicate that the aggregation of PVRNase is due to hydrophobic interactions of the covalently attached valine peptides. The utility of PVRNase aggregation as a model for studying hydrophobic interaction and the differences in the mode of denaturation by urea and guanidine hydrochloride are discussed.

In the attempts to study the forces involved in tertiary and quaternary structure of proteins, polypeptidyl proteins offer a convenient model (Becker and Stahmann, 1953) whose virtues reside in the fact that one may attach a variety of amino acids in varying amounts to a protein and then study the properties of the derivative with respect to the behavior of the attached peptides.

Ona (1959) observed that solutions of poly-*t*-leucyl-chymotrypsin which were turbid at room temperature cleared upon cooling. Polyvalyl-RNase (PVRNase)[1] undergoes a similar aggregation reaction and provides a model for studying hydrophobic interactions in protein systems (Krausz, 1963). PVRNase was found to exhibit reversible thermal aggregation at relatively low temperatures (30–40°); however, this property was not present in the native RNase or in polyglycyl-RNase (L. M. Krausz, personal communication). A preliminary study of PVRNase aggregation under hydrostatic pressure revealed large volumes of activation, $\Delta V^{\ddagger}$, which were suggestive of hydrophobic or apolar interactions (Kettman *et al.*, 1966). We wish to further examine this model system and to use it to address questions concerning the mechanism of protein denaturation by urea and guanidine hydrochloride, among other reagents.

Methods and Materials

RNase. Bovine pancreatic ribonuclease A was obtained from Sigma Chemical Co. (lot no. 958-0-0330). The enzyme was chromatographed on IRC-50 ion-exchange resin according to the procedure of Crestfield *et al.* (1963). The "A" component was preceded by an unexpected component present in about the same amount as "B." Only component A which corresponded to that reported in the literature was used in the modification reactions. The *N*-carboxyanhydride of valine (Val-NCA) was prepared from L-valine by the phosgene method of Berger *et al.* (1958) using anhydrous tetrahydrofuran instead of dioxane as the solvent.

Miscellaneous Reagents. *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) was obtained from Calbiochem. Tris(hydroxymethyl)aminomethane (Tris), glycylglycine (glygly), and urea were obtained from Sigma Chemical Co. The urea was recrystallized twice from ethanol and stored over P_2O_5 until used. Solutions of urea were made fresh each time and used within a day. Tetramethylammonium chloride (TMAC) and tetra-*n*-butylammonium bromide (TBAB) were Eastman White Label reagents. TBAB had to be recrystallized from hot benzene and petroleum ether (bp 30–60°) to remove the yellow material. Both TMAC and recrystallized TBAB were desiccated *in*

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[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: PVRNase, polyvalylribonuclease; Val-NCA, *N*-carboxyanhydride of valine; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TMAC, tetramethylammonium chloride; TBAB, tetra-*n*-butylammonium bromide; GuCl, guanidine hydrochloride; TMV A, the trimer (protein) subunit of tobacco mosaic virus.

vacuo over P_2O_5 for several days before using. Guanidine hydrochloride (GuCl) obtained from Matheson Coleman and Bell was recrystallized from ethanol–benzene and from methanol. All other chemicals were reagent grade and used as received.

PVRNase Preparation. Conditions for reacting RNase with Val-NCA were similar to those used by Becker and Stahmann (1953) except that pH 7.0 was used. The molar ratio of NCA to available amino groups was 5:1. RNase (322 mg) in 34.5 ml of 0.067 M phosphate buffer at pH 7.0 was cooled to 4°. To this was added 167 mg of Val-NCA as a powder. The admixture was stirred magnetically until completion of reaction (at least 8 hr). The resulting white, opaque solution was centrifuged for 10 min at 27,000*g*. The milky, thin supernatant fluid was concentrated to about 20 ml in a Diaflo cell using a UM-2 membrane (Amicon Corp., Cambridge, Mass.) at 4°. The solution was adjusted to pH 4.5 with 2 N HCl and then applied to a 2 × 38 cm phosphocellulose column with an exchange capacity of 17.2 mequiv (20 g of dry phosphocellulose) (Anfinsen *et al.*, 1962). The column was first eluted with 140 ml of 0.05 M citrate buffer at pH 4.4 containing 0.05 M NaCl to remove contaminating oligopeptides. This was followed by 0.2 M citrate buffer at pH 6.0 containing 0.2 M NaCl which eluted the PVRNase in the first ultraviolet-absorbing peak. Those fractions showing A_{280} greater than 0.1 were pooled and stored frozen.

PVRNase Characterization. Amino acid analyses were performed on a Beckman–Spinco Model 120B analyzer with accelerated chromatography systems. Samples for hydrolysis were sparged with nitrogen and sealed *in vacuo* into ampules. Because of the resistance of valine peptides to acid hydrolysis (Stracher and Becker, 1959) PVRNase and DNP-PVRNase samples were hydrolyzed in concentrated HCl at 110° for 16 hr. Dinitrophenylation of RNase derivatives was done essentially by the method of Fraenkel-Conrat *et al.* (1955). The difference in the valine content between DNP-PVRNase and PVRNase indicated the number of valine peptide chains attached to the RNase molecule.

The PVRNase was assayed with yeast RNA as the substrate according to Kalnitsky *et al.* (1959). The enzyme derivative was also assayed with cytidine 2′,3′-cyclic phosphate as the substrate according to Josefsson and Lagerstandt (1962).

Aggregation Kinetics. The scattering of light at 320 mμ was used as a measure of aggregation. The decrease in light transmission by the aggregating solution was followed in a Cary 14 spectrophotometer. The rectangular cuvets were held in a water-jacketed cuvet holder with brass inserts to assure efficient heat transfer. The cuvets were the semimicro type with approximately 1.5-ml capacity and 1-cm light path. Temperatures of the samples were recorded using a thermistor probe (Yellow Springs Instrument Co. Model 43) mounted inside the cuvet. In the usual procedure for an aggregation measurement the cuvet was first equilibrated at 39°, then the protein solution (which was kept on ice) was pipetted in, the thermistor was inserted, and the spectrophotometer and recorder were started. Measurements were usually made for 5 min. The temperature of the solution inside the cuvet reached 39° in about 4 min. Protein solutions contained 0.38 mg/ml unless otherwise stated.

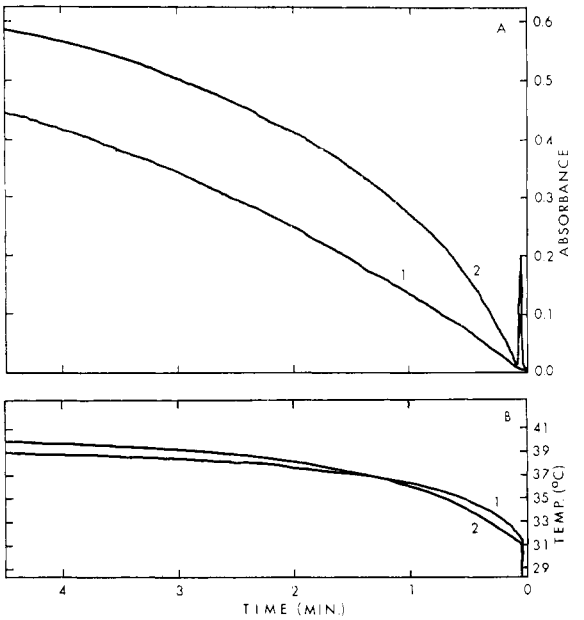

FIGURE 1: Some examples of the time course of aggregation. (A) The increase in aggregation with time has been measured as A_{320}. Both samples contained 0.36 mg/ml of protein in 0.01 M TES buffer at pH 7.4. Sample 1 was 0.01 M in NaCl and sample 2 was 0.75 M in NaCl. (B) These recorder tracings reflect the actual cuvet temperatures during the turbidity measurements.

The rate of aggregation was defined as $\Delta A_{320}/\Delta t$ (min). Rate values were obtained by taking the reciprocal of the slope resulting from plots of $1/\Delta A$ *vs.* $1/\Delta t$. Usually A values at 1, 2, and 3 min were used for the double-reciprocal plots. Where lag phases were encountered, A values at 2, 2.5, and 3 min were used.

Results

PVRNase Characterization. The enzyme derivative prepared for the following studies contained 22.8 moles of added valine/mole of protein at 7.6 sites, with an average chain length of 3.0 residues. The PVRNase preparation exhibited 71% of the activity of native RNase at pH 5.0 with yeast RNA as substrate, while 129% of unmodified RNase activity was detected at pH 6.8 with cytidine 2′,3′-cyclic phosphate. This suggests that at least some of the added valine peptides are located on lysine residues in the vicinity of the enzyme active site tending to sterically retard the hydrolysis of macromolecular but not low molecular weight substrates. Similar distinctions between small and macromolecular substrates have been noted for polyalanyl-Taka-amylase A by Isemura *et al.* (1964) and for polyalanyl-RNase A by Wellner *et al.* (1963).

Reaction Order for Aggregation. The apparent order of the aggregation reaction was examined at two pH values, above and below the pH of maximum rate.

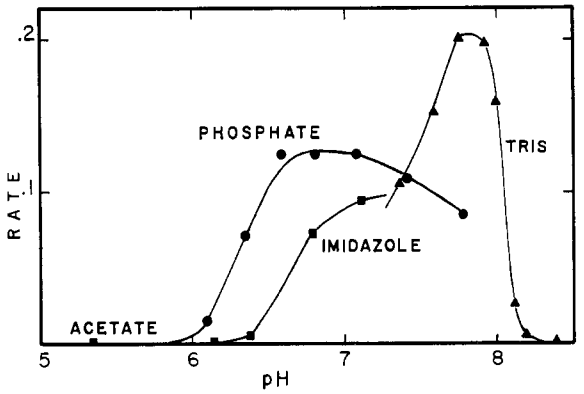

FIGURE 2: PVRNase aggregation rate as a function of pH and buffer type. All buffers were present at 0.05 M.

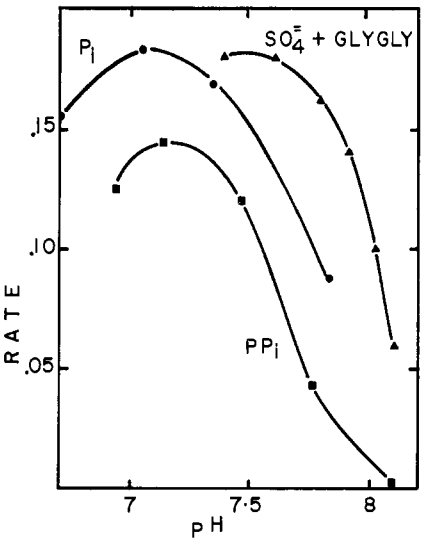

FIGURE 3: Effect of multivalent anions on aggregation. Electrolyte concentrations were 0.025 M sodium pyrophosphate, 0.05 M sodium phosphate, 0.05 M glycylglycine, and 0.05 M sodium sulfate. At pH 7.4 the calculated ionic strengths were 0.165, 0.175, and 0.23, respectively, for the three buffer systems.

No significant differences were found at pH 7.93 or 7.40 in TES buffers. The time-course plots were analyzed in two ways. The first was a fractional time method of Ansevin and Lauffer (1963). This procedure yielded values of $n = 1.65$–1.8 at pH 7.93 and $n = 1.65$ at pH 7.40.

The second method of analysis of time-course plots involved the estimation and use of initial rates of aggregation. The log (rate) *vs.* log (protein concentration) was plotted to obtain a straight line whose slope equalled n, the reaction order (Frost and Pearson, 1961). Values of $n = 1.6$ at pH 7.4 and $n = 1.65$ at pH 7.93 were obtained.

Time Course of Aggregation. Changes in A_{320} with time typically revealed a hyperbolic curve which, after a moderate initial rise, fell off rather markedly (Figure 1A). In general this pattern resembles that which is associated with a condensation polymerization (Oster, 1947; Ansevin and Lauffer, 1963). The marked falling off of the scattering (*i.e.*, A) with time reflects a decrease in the effective scattering as the average particle size dimensions exceed the wavelength relationship defined in Rayleigh scattering (Timasheff, 1966). The decline in the rate of scattering does not reflect attainment of a steady state nor a decline in the rate of aggregation. The time-course pattern is similar to that observed for TMV A protein aggregation (Ansevin and Lauffer, 1963) and bushy stunt virus salting out (Oster, 1947). It differs significantly from the time course of ovalbumin aggregation which pattern has been designated as addition polymerization (Tomimatsu 1965).

The time course for the temperature change within the sample cuvet is also shown in Figure 1B. Typically the temperature is about 33 or 34° at zero time and rises nonlinearly to 39° in 4.5 to 5 min. This complicates the interpretation of initial rates of aggregation with respect to the designation of the temperature at which the aggregation takes place and precludes any attempts of determining the energies of activation of the aggregation reaction.

The determination of the initial rate of aggregation has been difficult especially in those time-course curves where the shape of the curve as well as the extent of the rise in A varied with the change in the parameter being studied. The change in curve shape was noted only at pH values on the alkaline side of the rate maximum and at high GuCl concentration. This general problem was noted at length by Ansevin and Lauffer (1963) in the aggregation of TMV A protein. The empirical method used in this paper for handling aggregation rate data was devised to cope with the aforementioned problems. The procedure appears to normalize the data to a hyperbolic curve over some 3 min and also permits the estimation of very high aggregation rates. In those cases where the aggregation had proceeded rather substantially by the time the spectrophotometer and recorders were turned on, no really better way of estimating initial rates was at hand. In those situations where the shape of the time course plots permitted a reasonable estimation of the initial rate by a slope drawn toward the origin, rate values were obtained which agreed very well with values obtained by the double-reciprocal method.

pH Dependency of the Aggregation Rate. A study of the effect of pH on the rate of aggregation revealed that the reaction proceeds in a rather narrow range. Figure 2 shows the results of a study carried out in acetate, phosphate, imidazole, and Tris buffers. No aggregation was observed with a dialyzed sample of PVRNase at pH 5.5. The width of the pH range was seen to depend on the buffering ion. Clearly, in Tris buffer the reaction took place over a much narrower pH range in phosphate or imidazole. This unexpected observation was studied further.

Effect of Various Buffer Ions on Aggregation. Tris, TES, and glygly buffers were compared. While a weak cationic buffer such as Tris gave a sharp change in the aggregation rate with pH, the inclusion of a carboxylic group (glygly) in the buffering species did not seem to affect the pH–rate profile significantly. The presence

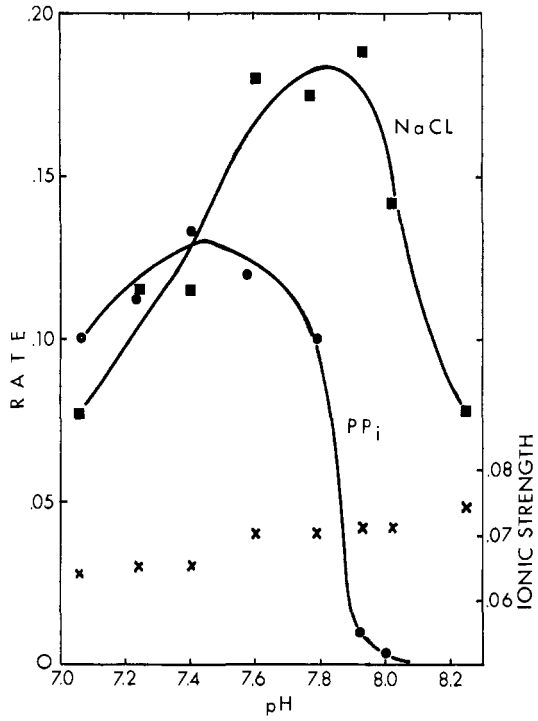

FIGURE 4: Effect of sodium pyrophosphate on aggregation. Both sets of samples were buffered with 0.01 M TES. Sodium pyrophosphate was present at 0.008 M. NaCl was added in varying amounts to adjust the ionic strength to that of the pyrophosphate samples at each point in pH.

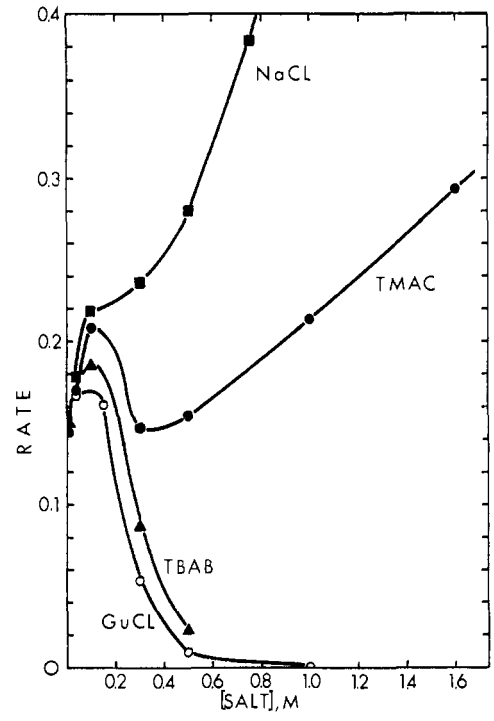

FIGURE 5: Effect of electrolytes on aggregation. Reaction was in 0.01 M TES at pH 7.4.

of sulfonate, a strong anion, in TES seemed also to have little effect on the aggregation profile.

Effect of Polyanions. The inclusion of only 0.05 M Na_2SO_4 into the reacting medium was observed to significantly lower and shift the rate maximum to acid pH. The rate profile was also broadened. This prompted the examination and comparison of phosphate and pyrophosphate. The results shown in Figure 3 indicate that from sulfate to phosphate to pyrophosphate there is a shift of the rate profile toward acid pH accompanied by a slight lowering of the maximum. Since the three curves in Figure 3 differed slightly in ionic strength a more careful study was conducted with sodium pyrophosphate in the presence of TES buffer, as shown in Figure 4. While the ionic strength varied slightly over the pH range studied, the solutions were adjusted so that at each point in pH the ionic strength in the two curves was the same. The presence of pyrophosphate anion is significant and since the effect is observed at rather low concentration, the effect on aggregation may be called specific (also see Ginsburg and Carroll, 1965).

Effect of Ionic Strength. Figure 5 shows the effect of increasing sodium chloride concentration on the aggregation rate at pH 7.4 in TES buffer at 0.01 M. The nonlinear pattern in the rate change suggests two regions of salt effects. In the low salt region (0–0.3 M) an increase in salt elicits a rapid rise in the rate which levels off. The high salt region (0.3 M) shows a rather gradual rise in the rate with salt concentration, and the rate change is logarithmic (see Figure 8).

Besides sodium chloride three other electrolyte, have been examined with respect to their effects on the aggregation rate. It can be seen in Figure 5 that all electrolytes have in common the effect of causing a sharp rise in the rate at low salt concentration. Above 0.15 M, however, the effects on rate vary markedly with the type of electrolyte. While increasing concentrations of NaCl increase the aggregation rate, the tetraalkylammonium salts show different effects depending on the length of the alkyl groups. TMAC increases aggregation only at somewhat higher concentration compared to NaCl, while TBAB distinctly decreases the rate at very low concentrations. Of the reagents examined, GuCl is the most effective in diminishing aggregation rate. Even though the inhibition pattern is like that of TBAB, the difference in the structures of the two compounds makes it unlikely that GuCl is affecting PVRNase aggregation by mechanisms similar to that of the alkyl salt.

Effect of Nonelectrolytes. Figure 6 shows that the aggregation is rather sensitive to the presence of urea. A tenfold drop in rate is seen in going from 0 to 4 M urea, with a midpoint in the rate change at about 2 M urea. This rate change occurs at somewhat lower concentration than the optical rotation change of chymotrypsin with a midpoint at 4 M urea (Martin and Bhatnagar, 1967) or of RNase with a midpoint at 6 M urea (Nelson and Hummel, 1962).

Studies of β-lactoglobulin in aqueous ethylene glycol solutions have indicated that this solvent does not severely affect the tertiary structure of the protein until very high concentrations of the glycol are used (Tanford *et al.*, 1962). It was of interest to examine the effects of this mild denaturant on the aggregation of

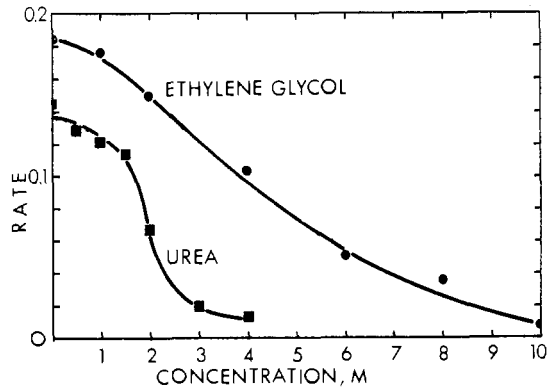

FIGURE 6: Effect of nonelectrolytes on aggregation. Reaction was in 0.01 M TES at pH 7.4.

PVRNase. Figure 6 shows that ethylene glycol is somewhat less effective than urea in diminishing aggregation. It should be noted, however, that at 10 M glycol (approximately 55% [v/v]) where aggregation rate of PVRNase is about 95% suppressed, the optical rotation and the Moffitt–Yang b_0 constant for β-lactoglobulin shows little change (Kientz and Bigelow, 1966).

Discussion

PVRNase Characterization and Aggregation. The enzymatic activity of the moderately modified RNase indicates that a significant amount of native structure has been retained in the derivative. The superactivity expressed with the cytidine cyclic phosphate may reflect some perturbation of the structure not involved in substrate binding but involved in catalysis (Becker and Sawada, 1963). We may then assume that our model consists of RNase A in its native conformation like that suggested by Kartha *et al.* (1967) but with valine peptides attached to its surface.

The results from the attempts to determine reaction order suggest a rather complex mechanism with n between $^3/_2$ and 2. The value obtained may be compared with the $n = 2$ for the TMV A protein aggregation system (Ansevin and Lauffer, 1963) which is similar in many respects to the PVRNase system. Unfortunately the PVRNase system has not yet been amenable to an adequate definition of its aggregation intermediates and products. The nonintegral order may result from a rate-determining step which involves the making available of the valine peptides for intermolecular interaction rather than from a multiple collision process. Alternatively, the observed order may be a resultant of the changes in temperature and the scattering factor during the time of turbidity rate measurement.

Effect of pH and Buffer Ions. In a titration study of polyvalyl-RNase containing 22 added valine residues/mole of enzyme, F. Sawada (unpublished results) observed that the isoelectric point (pI) of the derivative was about 7.8 compared to the native protein with pI = 9.6–9.8. This pI shift no doubt resulted from the conversion of most of the ϵ-amino groups into α-amino groups upon polypeptidylation. Sawada also observed in PVRNase solutions a pH-dependent turbidity which was not seen in native RNase solutions. The maximum turbidity was seen between pH 7.0 and 7.5, which nearly coincided with the pI. These observations were essentially verified in the present experiments except that a pronounced sensitivity of the aggregation to certain types of buffering ions and multivalent anions was seen. This effect of the anions on the aggregation was unexpected, although Hummel and coworkers (Nelson *et al.*, 1962) had reported that the presence of these ions could retard the urea denaturation of RNase A. They had observed that the order of effectiveness in protecting RNase from urea denaturation was sulfate < phosphate < pyrophosphate. Furthermore, the pH dependency of the protective effects (maximum about pH 5) was interpreted to mean that the anion interaction with RNase was a specific one which prevented unfolding of the tertiary structure. With the PVRNase aggregation system other studies had led us to believe that the rate was mainly dependent on the length of the attached valine peptides (Krausz *et al.*, 1962). The results with the multivalent anions suggest that in their presence, PVRNase, due to ion binding (as suggested by the low salt concentration effects), may undergo a shift in its isoelectric point but more importantly it may undergo a conformational change significant enough to bring about a reduction of the aggregation. From the three-dimensional model of RNase A by Kartha *et al.* (1967) it is not hard to see the source of a conformational shift in the pincer-like action described by Bello (1968) between the nonpolar and polar halves of this kidney-shaped molecule when it interacts with the substrate or a multivalent anion.

There appears to be a strong similarity between the PVRNase system and the TMV A protein system with respect to the sharp dependency of the aggregation rate on pH as well as the narrow pH range where aggregation occurs. Both systems exhibit a maximum near their respective isoelectric points (pI = 3.5 for TMV A protein). The precise reasons for this sharp dependency on pH are not known for either system.

Effect of Electrolytes. The effect of salts on the behavior of proteins in solution has been the concern of many studies (von Hippel and Wong, 1965; Martin and Bhatnagar, 1967; Nagy and Jencks, 1965). Electrolyte addition to protein solutions can result in a number of effects on the interaction of protein with water, on the liquid structure of water, and on the interaction of polar functional groups of the protein with the added solute itself (Robinson and Jencks, 1965a,b; Frank and Wen, 1957; Klotz, 1966). Besides the effect of ionic and polar functional groups, added electrolytes can (although by different mechanisms) affect the solubility of nonpolar (aliphatic and aromatic) groups in proteins. Because of their substantial numbers in most proteins, the response of these nonpolar groups to the added electrolytes predicates the solubility or insolubility of the protein in water. As Nozaki and Tanford (1963) have shown, these nonpolar functional groups can show a wide range of values for a given thermodynamic function such as ΔF_{tr} (the free energy of transfer of an amino acid side chain from pure water to a mixed aqueous solvent). Thus, in the use

of polypeptidyl proteins as model systems, the selective use of a particular *type* of nonpolar amino acid is as important as whether or not it is nonpolar. Rather distinct differences in their behavior toward aqueous solvents have been noted between glycine or alanine and valine or leucine (Nozaki and Tanford, 1963). Hence the resistance of polyglycyl-RNase to thermal aggregation was not unexpected although its superresistance to such treatment was (Krausz, 1963). The work by Anfinsen and colleagues (1962) on polyalanyl-RNase has never indicated any solubility problems with even extensively modified RNase. Polytyrosyl-RNase, on the other hand, exhibited solubility problems in all but the most lightly tyrosinated preparations. Polyvalyl-RNase shows intermediate properties between that of polyglycyl- and polytyrosyl-RNase. Lightly modified PVRNase (approximately 9 moles/mole of enzyme) does not readily aggregate at 30–40° (Krausz, 1963), while more heavily modified (20 or more moles of valine per mole of enzyme) preparations do. Also because of the nature of the added peptides in PVRNase, the thermally induced aggregation with this derivative has been attributed to hydrophobic or apolar interactions.

The two distinct patterns of the response of the aggregation rate to salt concentration indicate the presence of at least two kinds of interaction phenomena arising from the presumed valyl–valyl interactions of PVRNase. In the lower salt concentration range we have been assuming that it is the NH_3^+ to NH_3^+ repulsion being diminished by the added salt, resulting in an acceleration of aggregation. This interpretation obtains from the plot in Figure 7 of the log (rate/rate$_0$) *vs.* $\sqrt{\mu}$ (where μ = ionic strength) according to the equation by Brønsted and Bjerrum (Glasstone *et al.*, 1941)

$$\log k = \log [k_0] + 1.02 Z_a Z_b \sqrt{\mu}$$

where Z_a and Z_b are the charged groups involved in the reaction. Instead of k and k_0, the reaction constants with and without added salt, respectively, we have used the corresponding observed rates. The slope obtained from the plot yields a Z_aZ_b product of +0.725. Assuming that Z_a and Z_b are both equal and positive, the square root of 0.725 yields Z_a or Z_b = +0.852. Now assuming that the α-amino pK = 8.10 (as for the tripeptide Gly-Ala-Aly; Edsall and Wyman, 1958b) then at pH 7.4, where the aggregation reaction was carried out, the Henderson–Hasselbach equation shows the α-amino group to possess a +0.83 charge. This value corresponds very well with the +0.85 charge calculated for Z_a or Z_b. In view of the fact that PVRNase is a polyelectrolyte, the apparent Brønsted behavior of the aggregation (even at a pH where the net charge on the protein is about zero) is striking. Because of reservations indicated earlier as to the quantitative nature of the rate data, no strong emphasis is placed upon the good agreement obtained. Nevertheless, the results are of interest, and tend to strengthen the notion that the thermal aggregation of PVRNase

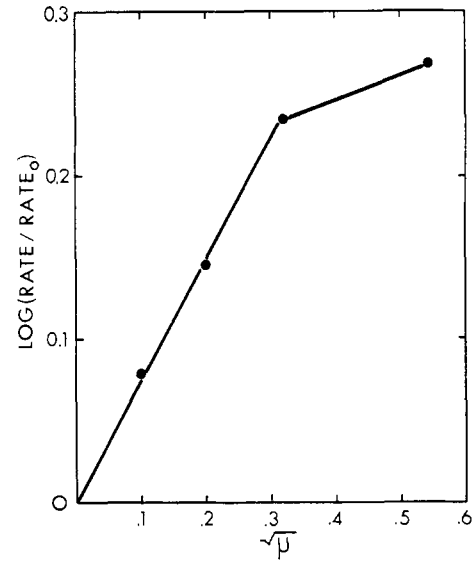

FIGURE 7: Relative aggregation rates as a function of ionic strength. Buffer was 0.01 M TES, pH 7.4. NaCl was the electrolyte.

results from the intermolecular interactions between the attached valine chains. Furthermore, these results at low salt concentration suggest interactions of the added electrolyte with the loci for aggregation in PVRNase.

In the effects of higher salt concentration on the aggregation rate, a casual examination of the data would suggest that something other than simple electrostatic repulsion is being overcome to accelerate aggregation. Accepting now that the driving force in these aggregations is the gain in solvent entropy (Lauffer, 1964) which would accrue upon the intermolecular interactions of the valine peptides attached to PVRNase, one may expect that the effect of higher salt concentrations on the aggregation rate is one of accelerating a salting-out effect. This may be verified as follows. In describing the salting out of simple nonelectrolytes from aqueous solutions, an empirical expression has been long used (Edsall and Wyman, 1958a): log $[S_2/S_2^*] = -k_s m_3$, where S_2 and S_2^* correspond, respectively, to the solubility of the nonpolar compound in the presence and absence of the electrolyte (m_3) and k_s is a constant which is dependent on the nature of the electrolyte. We now assume that the thermodynamics of the reactants and activated complex behaves like that of solubility of proteins, and then substitute aggregation rate data in place of solubility (S) and plot log [rate/rate$_0$] *vs.* [salt]. Figure 8 shows this treatment for data obtained in the presence of NaCl, TMAC, TABA, and GuCl. It can be noted that above 0.1 M salt straight-line relationships are obtained. NaCl definitely salts out PVRNase. TMAC salts out the protein at higher concentrations. By contrast TBAB and GuCl salt in the PVRNase. It would appear then that the response of the aggregation rate to salt concentrations above 0.1 or 0.15 M involves solvent-mediated effects or at least not a direct interaction with the α-amino groups of the attached valine peptides.

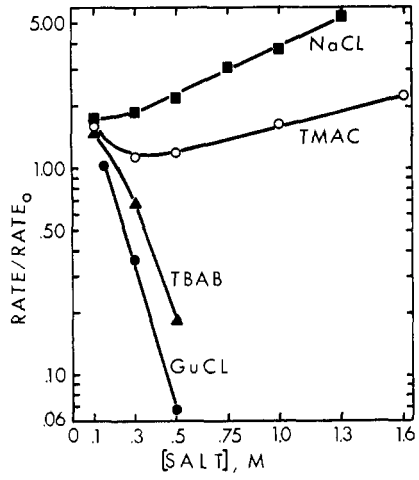

FIGURE 8: Logarithm of rates as a function of salt concentration.

Although it has been pointed out that TMA^+ ion can salt-in nonpolar compounds while having no effect on proteins (Robinson and Jencks, 1965b) the marked difference noted in the effects of TMAC and TBAB on aggregation is in line with some recent observations which suggest that the tetramethylammonium ion is a structure breaker (Boyd *et al.*, 1967; Kay *et al.*, 1966; Bunzl, 1967).

Guanidine hydrochloride is the most effective reagent thus far examined in inhibiting PVRNase aggregation. A strong interest in this denaturant has centered around the question: Do GuCl and urea denature proteins by the same mechanisms (Wetlaufer *et al.*, 1964)? The similarity in the net changes of several experimental parameters for proteins in both urea and GuCl have been accepted to mean that these reagents evoke conformational changes by similar mechanisms (Martin and Bhatnagar, 1967). But while the final states attained by a protein in these solutions may be similar, GuCl solutions usually affect the transitions at one-half to one-fourth the molar concentration of urea solutions. This suggests that the route or mechanism by which conformational transitions in proteins take place may be different in the presence of GuCl and urea even though the final states attained may be similar. The difference in the behavior of PVRNase aggregation in GuCl compared to urea in the 0–0.1 M range also serves to distinguish the effect of these reagents on proteins. Even above 0.15 M, the salting in by GuCl takes place at such relatively low concentrations that it may be interacting directly with the valine peptides on the protein derivative (see Robinson and Jencks, 1965a). Alternatively, GuCl may perturb the structural organization of liquid water in a manner distinct from urea such that the effects are manifested on protein solubility at even low concentrations of salt.

In the study of urea and GuCl as protein denaturants and of the forces important in native protein structure, the thermal aggregation of PVRNase is a unique model. First, the loci of intermolecular interactions involve functional groups which are naturally found in proteins. Unlike the synthetic homopolypeptides and block copolymers, these lightly modified RNase derivatives do not exhibit highly reinforced cooperative effects (Auer and Doty, 1966). Finally, PVRNase is a derivative in which the amount of hydrophobic groups has been increased over the original protein by attaching valine residues to the surface. This location has forced the apolar side chains of valine to interact with the solvent rather than to reside in the preferred nonpolar environment of the protein interior (Kendrew *et al.*, 1961). With the charged α-amino group still at the ends of the added valine peptides, one has at hand a model system whose properties reflect a balance of electrostatic and hydrophobic interactions. It is this apparently sensitively poised property which has enabled the distinction between urea and GuCl effects.

Given the proper pH, the PVRNase system which aggregates at moderate temperature demonstrates the importance of hydrophobic groups in the tertiary and quaternary levels of the structural organization of proteins. In this regard the hydrophobic residue percentage index of van Holde (1966) is interesting in that it accurately predicts that where more than 20 valines have been added to RNase this protein should aggregate. The preparation studied here contained 23 valines. Other preparations containing 11 and 17 added valines have not been observed to aggregate under the same conditions (A. H. Nishikawa, unpublished results). The "average hydrophobicity" parameter of Bigelow (1967) also predicts aggregation for the PVRNase with 23 added valines.

The effect of ethylene glycol on PVRNase aggregation is even more gradual than that of urea, which suggests that it interacts indirectly with the protein *via* a solvent-mediated change in chemical potential (Klotz, 1966). It would have been of interest to examine the effects of ethylene glycol at concentrations higher than 10 M, but the effect of the reagent on the native tertiary structure probably would have obscured any effects of the added valine peptides.

These studies suggest that the response of PVRNase to various solvent media reflects the involvement of both electrostatic and nonpolar (or hydrophobic) forces. The Brønsted–Bjerrum treatment of aggregation rates and ionic strength points to electrostatic repulsions at the α-amino groups (of the attached valine peptides) which can be overcome at low salt concentrations. The increase in aggregation at high concentrations of NaCl or TMAC and the decrease at high levels of urea, TBAB, or GuCl all suggest that the aggregation of PVRNase involves hydrophobic (or nonpolar) intermolecular interactions of the attached valine peptides. The acceleration of aggregation at low electrolyte concentrations suggests a difference between urea and GuCl in their interactions with protein, hence in their modes of protein denaturation. The PVRNase aggregation system suggests that nonelectrolytes affect protein structure by an indirect means through the solvent medium. Electrolytes may perturb protein structure directly or indirectly depending on their chemical nature.

The quantitative study of PVRNase aggregation

has been fraught with technical difficulties and so the experiments presented here must be considered somewhat qualitative. In spite of these reservations, the responses of the PVRNase aggregation to systematic changes in the solution environment indicate that this protein derivative is a useful and consistent model for studying the hydrophobic interactions involved in protein structure.

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