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Title PREPARATION AND COMPARATIVE SOLUBILITY OF POLY-L-TYROSYL LYSOZYME AND POLY-O-METHYL-L-TYROSYL LYSOZYME

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In order to examine some possible tyrosyl interactions, poly-tyrosyl lysozyme and poly-O-methyltyrosyl lysozyme were prepared and their solubilities were compared. Both derivatives exhibited similar pH dependent solubilities, showing a minimum solubility at about pH 8. Their solubilities varied with temperature, having a positive enthalpy of solution. The effect of a lyotropic series of ions on the solubility of these lysozyme derivatives followed the same order in their ability to solubilize these derivatives as their ability to disaggregate proteins. The tyrosyl and O-methyltyrosyl lysozyme aggregations are believed to be due to interactions which cannot be delineated as singly hydrophobic, ionic or polar interactions.

PREPARATION AND COMPARATIVE SOLUBILITY OF POLY-L-TYROSYL LYSOZYME AND POLY-O-METHYL-L-TYROSYL LYSOZYME

by

LYNN CORRINE VENIER

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

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To my parents and husband. . .

APPROVED:

Redacted for Privacy

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Typed by Marion F. Palmateer

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INTRODUCTION

In an attempt to understand the forces involved in the association of proteins and the maintenance of tertiary structure of proteins, the conditions involved in aggregating or dissociating proteins can be investigated. If one modifies a protein by adding peptide residues of a single amino acid, a comparison of the solubilities of this modified protein to that of the native protein would indicate the interactions of the added peptide residues. Becker (1962) has shown that the changes in enzymatic and physical properties of polypeptidylated chymotrypsin and ribonuclease from those of the native were due to the added peptides. In the study of polypeptidyl proteins as model systems, there is the assumption that the reaction conditions for polypeptidylation are mild enough not to alter the native conformation of the molecule. Indeed, it has been shown by Epstein and others (1962), Anfinsen, Sela and Cooke (1962) and Becker (1962) that the structural features of many polypeptidyl proteins remain unchanged from those of the native protein.

The possibility remains that the forces involved in the association and dissociation of proteins may not be the same, but as reasoned by Nagy and Jencks (1965) each is of interest in its own right,

and elucidation of interactions which cause dissociation of proteins provides an indication of, if not a direct demonstration of forces involved in maintaining quaternary and tertiary structure.

In this study, lysozyme was chosen for investigation and modification for a number of reasons. It is a compact, globular protein, reasonably stable to a variety of solutions and temperature variations (Jollés, 1964), and its secondary and tertiary structures have been confirmed with X-ray analysis (Blake et al., 1965). Lysozyme contains six lysine residues, one of which is N-terminal (Jollés, 1964), providing seven sites for the attachment of amino acid residues by reaction of ϵ - and α - amino groups with an N-carboxy-amino-acid anhydride.

The non-covalent interactions of tyrosyl residues are of special interest not only because of their involvement in the internal hydrophobically bonded areas of lysozyme (Blake et al., 1965) and their participation in carboxylate interactions in ribonuclease (Li, Riehm and Scheraga, 1966), but also because of their role in the reversible association of carboxypeptidase A (Bethune, 1965), polytyrosyl trypsin (Glazer, 1962) and polytyrosyl acetamidinated ribonuclease (Lynn, 1966).

In order to elucidate the nature of tyrosyl interactions, a prime consideration is the extent of involvement of the phenolic hydrogen in hydrogen-bonded interactions. By comparing the behavior

of the tyrosyl peptides to a peptide in which the phenolic hydrogen has been replaced by an alkyl group, one can ascribe certain differences as being due to the elimination of hydrogen bonding with the tyrosyl side chain as a donor. O-Methyltyrosine appears to be a reasonable analogue for tyrosine for this purpose, and has been used to investigate the extent to which tyrosyl phenolic hydrogen-bond donation affects the ultraviolet difference spectra in proteins and amino acids (Wetlaufer, Edsall and Hollingworth, 1958).

With this possibility of eliminating tyrosyl hydrogen-bonded interactions, it seemed reasonable to prepare and investigate the solubility properties of both polytyrosyl lysozyme and poly-O-methyltyrosyl lysozyme.

EXPERIMENTAL

Preparation of N-Carboxy-L-Tyrosine Anhydride (Tyrosine NCA)

Tyrosine NCA was prepared following the method of Berger et al. (1958) with improvements developed in this laboratory (Lynn, 1966). L-Tyrosine, Calbiochem lot no. 36337 (2 gm) which had been dried in vacuo at 100° for one day was suspended in anhydrous, freshly distilled tetrahydrofuran (100 ml). Dry phosgene was bubbled through this suspension with constant stirring at 40°. When the reaction was judged complete by clarification of the solution (one to two hours), the reaction mixture was cooled below room temperature and flushed with dry nitrogen gas to remove excess phosgene. The tyrosine NCA solution was concentrated to a crystalline slush under reduced pressure at room temperature. To this was added dried, freshly distilled ethyl acetate (20 - 30 ml). The insoluble fraction, precipitate I, was removed by filtration. Freshly distilled, dried petroleum ether, b. p. 40-44° (120 ml) was added to the filtrate, precipitating the tyrosine NCA, precipitate II. Both the precipitates decomposed at about 190°. (Literature value 195°, Berger, et al. 1958). Infrared spectra of both precipitates showed no amide bands in the 1650-1700 cm^{-1} region and did show strong anhydride bands at 1830 cm^{-1} and 1760 cm^{-1} (Nakanishi, 1962, p. 45-46).

Preparation of O-Methyl-L-Tyrosine (O-Methyltyrosine)

O-Methyltyrosine was prepared following the procedure of Siedel, Sturm and Geiger (1963), with modifications found necessary to purify products.

L-Tyrosine, Calbiochem lot no. 36337, (50 gm) was suspended and partially dissolved in 2 N NaOH (142 ml) and H_2O (83 ml). This mixture was cooled in an ice bath and stirred continuously during the simultaneous, dropwise addition of acetic anhydride (67 ml) and 2 N NaOH (667 ml). The resulting clear solution was stirred for one hour at room temperature. This solution was acidified with 6 N H_2SO_4 (280 ml) and chilled to 4°. N-Acetyl-L-tyrosine was extracted from the resulting precipitate first with acetone, and then with 80 percent acetone. The N-acetyl-L-tyrosine was concentrated to a heavy syrup by evaporation of acetone under reduced pressure at room temperature.

N-Acetyl-L-tyrosine was dissolved in 30 percent NaOH (133 ml) and treated slowly, with stirring at room temperature,with Me_2SO_4 (63 gm). The reaction mixture was stirred for one-half hour at 60°, cooled and acidified with dilute H_2SO_4. The product, both a heavy oil and a crystalline product,was collected by filtration. The resulting oil and precipitates were then extracted with acetone and concentrated under reduced pressure at room temperature to a heavy glass

and recrystallized from hot water. The colorless crystalline plates had a melting point 140-142. 5° (uncorrected), (literature value, 146-147°; Siedel, Sturm and Geiger, 1963). The infrared spectrum shows strong absorption at 1610 cm^{-1} and 1700 cm^{-1}, corresponding to amide absorption, and peaks at 1030 cm^{-1} and 1240 cm^{-1} corresponding to aromatic ether absorption (Nakanishi, 1962, p. 36, 45, 46).

The N-acetyl-O-methyl-L-tyrosine (approximately 20 gm) was suspended in 4 N HCl (410 ml) and refluxed for two and one-half hours. The solution was concentrated under reduced pressure to a partially crystalline slush, cooled to 0° and filtered. The precipitated O-methyl-L-tyrosine hydrochloride was dissolved in a minimal amount of hot water and O-methyl-L-tyrosine was precipitated by neutralizing the solution with dilute NH_4OH. The precipitate, m. p. approximately 133°, could not be purified by further acidification and reprecipitation. By dissolving the precipitate in a minimal volume of hot water, treating with activated carbon, filtering and slow cooling, a white crystalline product was obtained, m. p. 243-244° (dec.)(corrected), (literature value, m. p. 243-244°; Siedel, Sturm and Geiger, 1963). By amino acid analysis, this product was shown to be chromatographically pure of any other ninhydrin-positive compounds. Infrared absorption spectra showed aromatic ether absorption at 1020-1030 cm^{-1} and 1220 cm^{-1} and the absence of the

amide band at 1700 cm^{-1} (Nakanishi, 1962, p. 36, 45, 46). Optical rotation, two percent in 1 N HCl gave $[\alpha]_{546}^{30°} = -7.35°$ (Literature value, $[\alpha]_{546}^{29°} = -5.9°$; Clark and Behr, 1932).

Preparation of N-Carboxy-O-Methyl-L-Tyrosine Anhydride (O-Methyltyrosine NCA)

O-Methyltyrosine NCA was prepared in a similar manner to tyrosine NCA. O-Methyl-L-tyrosine (2 gm) which had been dried *in vacuo* at 100° over P_2O_5 for two days was suspended in anhydrous, freshly distilled tetrahydrofuran (135 ml). This suspension was treated with dry phosgene with constant stirring at 40°. After two hours, the reaction mixture had clarified, and was cooled to room temperature and flushed with dry nitrogen to remove excess phosgene. O-Methyltyrosine NCA solution was concentrated under reduced pressure at room temperature to a crystalline slush. The O-methyltyrosine NCA was then dissolved in freshly distilled, anhydrous ethyl acetate (30 ml) and filtered quickly. To the filtrate, freshly distilled, dry petroleum ether, b. p. 40 - 44° (150 ml) was added. The resulting fine crystalline needles were removed quickly by filtration. A second crop of crystals was obtained by the addition of further petroleum ether (50 ml). Infrared spectra of each precipitate were identical, showing strong anhydride bands at 1750 cm^{-1} and 1850 cm^{-1} and showing the absence of any amide bands in the 1610-1700 cm^{-1} region (Nakanishi, 1962, p. 45, 46). Elemental analyses

showed C = 59. 61 percent, 59. 60 percent; H = 5. 26 percent, 5. 41 percent; N = 7. 06 percent; O = 29. 6 percent. Theoretically, for O-methyltyrosine NCA, C = 59. 72 percent; H = 5. 01 percent; N = 6. 33 percent; O = 28. 93 percent. The corrected melting point for O-methyltyrosine NCA is 183. 5° (dec.).

Preparation of Poly-L-Tyrosyl Lysozyme (PT-Lysozyme)

Preliminary studies to determine the degree of modification of lysozyme with varying molar ratios of added tyrosine NCA to lysozyme were carried out under the following conditions. Lysozyme (Worthington LYSF 635, twice crystallized, salt-free) (100 mg) was dissolved in 0. 1 M phosphate buffer (10 ml) pH 7. 52. These solutions were cooled to 4° and stirred rapidly during the addition of tyrosine NCA as a dry solid. The resulting suspension was maintained at 4° with constant stirring for one day, then centrifuged at 54, 000 x g for 60 minutes to remove the precipitate. The supernatant was dialyzed in no. 18 cellulose tubing against distilled water. Amino acid analyses showed that an added molar ratio of tyrosine NCA to lysozyme in the reaction mixture of 8. 1, 16. 2, and 81. 2 resulted in 2. 4, 3. 4 and 8. 0 added tyrosyl residues respectively per mole of lysozyme.

A similar preparation of polytyrosyl lysozyme was carried out in carbonate buffer, pH 8. 0, μ = 0. 10 with an added molar ratio of tyrosyl NCA to lysozyme of 35. 0. It was found that greater than

98 percent of the PT-lysozyme precipitated under the reaction conditions, but the product was soluble at a pH less than three or greater than nine. The PT-lysozyme used for solubility studies was prepared under the same conditions. Lysozyme (Worthington LYSF 638, twice crystallized, salt-free) (1 gm) was dissolved in carbonate buffer, μ = 0.10, pH = 8.0 (100 ml). The solution was cooled to 4° and stirred rapidly during the addition of 35 moles of tyrosine NCA per mole of lysozyme (0.510 gm). The resulting suspension was maintained at 4° with constant stirring for one day. The entire reaction mixture, including a bulky, white precipitate was dialyzed in no. 18 cellulose tubing against 0.01 N HCl. The remaining small precipitate was removed by centrifugation at 96,000 x g for 45 minutes. PT-lysozyme in the supernatant solution was concentrated by the following fractionation. The solution was made 0.05 M in citric acid by the slow addition of solid citric acid. The pH of the solution was then slowly adjusted to 4.55 by the addition of dilute NaOH, and the precipitated PT-lysozyme removed by centrifugation at 78,000 x g for 75 minutes. A suspension of the precipitate in 0.05 M citrate buffer was dissolved on bringing the solution to pH 2.95 by the addition of dilute HCl.

Preparation of Poly-O-Methyl-L-Tyrosyl Lysozyme (POMT-Lysozyme)

In order to approximate the degree of modification of lysozyme with O-methyltyrosine NCA to that of PT-lysozyme, varying molar ratios of O-methyltyrosine NCA to lysozyme were employed. Three samples of lysozyme (Worthington LYSF 638, twice crystallized, salt-free)(100 mg each) were dissolved in carbonate buffer, pH 8.0, μ = 0.10 (10 ml each). The solutions were cooled to 4° and stirred rapidly during the addition of O-methyltyrosine NCA. After one day of constant stirring at 4° the reaction mixtures were dialyzed in no. 18 cellulose tubing against 0.01 N HCl. The solutions were centrifuged first at 22,000 x g for three hours, then at 96,000 x g for 45 minutes. Amino acid analyses of each supernatant solution indicated that for the addition of 12.6, 21.0 and 35.0 moles of O-Methyltyrosine NCA per mole of lysozyme, 9.0, 16.0 and 21.7 moles of O-methyltyrosine per mole of lysozyme were added respectively.

The POMT-lysozyme used for solubility studies was prepared under the same conditions, using lysozyme (Worthington LYSF 645, twice crystallized, salt-free) (1 gm) in carbonate buffer, pH 8.0, μ = 0.10 (100 ml) reacted with a molar ratio of O-methyltyrosine NCA to lysozyme of 21.0 After reaction and dialysis as before, the supernatant solution obtained on centrifugation at 20,000 x g for one hour was concentrated (to less than 20 ml) in a Diaplex Ultrafil model

50 cell with a UM-2 membrane (Amicon Corp.).

Purification of PT-Lysozyme and POMT-Lysozyme

Both derivatives were purified in the same manner. The protein was dialyzed in no. 18 cellulose tubing against buffer I, a solution of 4 M urea, 0. 20 M NaCl, 0. 05 M citrate buffer, pH 3. 80, and applied to a phosphocellulose column (40 x 2 cm). The column was eluted with a linear gradient of equal volumes (250 ml) of buffer I and buffer II, a solution of 4 M urea, 0. 60 M NaCl, 0. 05 M citrate buffer, pH 5. 45. However, an aliquot of buffer I was allowed to deliver prior to the mixing of buffers I and II. The protein was eluted as a single, somewhat asymmetric peak starting at pH 4. 0 with POMT-lysozyme and at pH 4. 2 with PT-lysozyme. Each was dialyzed against 0. 01 N HCl, concentrated in a Diaplex Ultrafil model 50 cell with a UM-2 membrane (Amicon Corp.), and redialyzed against 0. 01 N HCl, pH 2. 3 for at least 24 hours.

Dinitrophenylation of PT-Lysozyme and POMT-Lysozyme

Solutions of lysozyme derivatives containing 3 to 5 mg protein were placed in 5 ml hydrolysis ampules and diluted to 1 ml with distilled water. Sodium bicarbonate (50 mg) was dissolved in the protein solution, followed by the addition of 1 ml of five percent dinitrofluorobenzene in 100 percent ethanol. The flask was then

wrapped in aluminum foil to exclude light, and was shaken mechanically at room temperature for six hours. The yellow precipitate thus obtained was washed twice with each water, ethanol and ether and recovered by centrifuging in a clinical centrifuge.

Hydrolyses of Lysozyme Derivatives and Their Amino Acid Analyses

PT-Lysozyme, POMT-lysozyme and their dinitrophenylated derivatives were hydrolyzed by the following procedure. From 2 to 5 mg of the protein in a 5 ml hydrolysis ampule were suspended in constant boiling HCl (2 ml). After flushing the ampule with nitrogen, it was sealed under vacuum and placed in a refluxing toluene bath at 110°. After 24 hours, the ampule was removed and the tip broken off. The HCl was evaporated under reduced pressure, and the residue dissolved in citrate buffer, pH 2. 2. Amino acid content of the hydrolysate was analyzed with a Beckman Spinco Automated Amino Acid Analyzer (Spackman, Stein and Moore, 1958). The ϵ -DNP lysine content of the hydrolysate was determined by the method of Nishikawa (1966) on the same amino acid analyzer.

Enzymatic Assay of Lysozyme and PT-Lysozyme

Assay of enzymatic activity of the preliminary preparations of PT-lysozyme were made observing lysis of Micrococcus lysodeikticus

cells by the change in optical density of the cell suspension at 450 mμ. The assay used is a modification of the method of Shugar (1952). A suspension of dried Micrococcus lysodeikticus from Worthington Biochemical Corp. of 0. 1 mg/ml was prepared in 0. 1 M phosphate buffer, pH 7. 0. Enzyme solutions assayed were approximately 0. 0188 mg/ml in 0. 15 M KCl, pH 6. 0. One-tenth ml of enzyme solution was mixed with 2. 9 ml cell suspension at zero time. The change in optical density of the mixture at 450 mμ against distilled water was expanded instrumentally on a Beckmann Model DB spectrophotometer with scale expansion, and recorded as a function of time. The change in optical density was linear within the first three minutes of the assay. Enzymatic assay under these conditions indicated that PT-lysozymes which contains 2. 4 or 3. 4 added tyrosyl residues are of about the same molar activity as lysozyme, but that more heavily modified lysozyme is of reduced activity.

In order to assay the activity of the PT-lysozyme used in the solubility studies, both the cells and the enzyme were in 0. 05 M citrate buffer, pH 3. 1. The activity of both the native lysozyme and the PT-lysozyme were of such a low level that differences in activity were not meaningful.

Solubility Studies

The solubility of lysozyme, PT-lysozyme, and POMT-lysozyme

in a particular solution was measured by the absorption of the supernatant solution at 282 mμ. Two ml of the test solution were placed in a 5 ml shell vial with 0. 2 ml of the protein solution. In the study of the effects of different salts, the solutions were made such that the final protein solution was at the concentration being studied. These solutions were sealed and equilibrated with constant shaking at 25°. Aliquots of the supernatant solution were removed with fritted glass immersion tubes by allowing the supernatant to diffuse in the tube. Samples thus removed were diluted one to ten with 0. 01 N HCl prior to absorption measurement.

Instruments and Chemicals

Carbon, hydrogen and nitrogen analyses were done by Elek Miroanalytical Laboratories, Torrance, California.

Oxygen analysis was done by Mr. Roger Scriven and Mr. Leslie Heasley, Oregon State University, Corvallis, Oregon.

Ultraviolet and visible spectra were determined with a Beckman DB recording spectrophotometer with 1-cm cells.

Infrared spectra were recorded on a Beckmann IR 8 spectrophotometer.

Optical rotation was determined with a Perkin-Elmer Model 141 polarimeter by Mr. George B. Large.

pH Measurements were made with either a Corning pH Meter

Model 12, or with a Beckman Zeromatic pH Meter.

Phosphocellulose used was Sigma Cellulose Phosphate Cation Exchanger, medium mesh.

Visking cellulose tubing was used throughout.

Other chemicals, unless otherwise stated, were of reagent grade and used without further purification.

RESULTS AND DISCUSSION

Modification of Lysozyme with Tyrosine NCA and O-Methyltyrosine NCA

Amino acid analyses of purified PT-lysozyme and its dinitrophenylated derivative have shown that about 16. 5 tyrosyl residues have been added to lysozyme, and that three ϵ - amino lysine residues remain unreacted with tyrosine NCA. With a maximum of four possible sites of attachment of the tyrosyl residues, the average chain length of added polytyrosine is 4. 1.

Since, under the hydrolysis conditions employed, 86 percent of the O-methyltyrosine is hydrolyzed to tyrosine, added O-methyltyrosyl residues are calculated as both the added tyrosine and O-methyltyrosine. Analysis of the purified POMT-lysozyme shows 10. 1 residues added at 5. 8 sites, giving an average chain length of 1. 7 O-methyltyrosyl residues.

A comparison of PT-lysozyme and POMT-lysozyme shows that the degree of modification of lysozyme in each case is of the same order of magnitude, but that there is some difference in the number of ϵ - amino sites reacted and consequently in the average chain length of the added residues. That these differences reflect a difference in availability of the ϵ - amino sites to either NCA is highly conjectural, especially considering the biphasic nature of the reaction.

As easily, one could invoke slight differences in buffer solutions, crystalline size or structure of either NCA in the suspension, or different rates of reaction of either NCA with ϵ - or α - amino sites.

Regardless, the function of POMT-lysozyme as a comparative model for PT-lysozyme interactions is still valid. Many similarities or differences in solubilities of these derivatives can be reasoned as being a function of the type of added peptides rather than a function of degree of modification of lysozyme.

Temperature Dependent Solubility of PT-Lysozyme

The increase in solubility of PT-lysozyme with temperature can be seen in Figure 1. From 0° to 41° a linear increase in solubility with temperature can be seen, indicating that for this temperature range the enthalpy of solution, ΔH, is positive. If the aggregation of PT-lysozyme were simplified to the equilibrium,

$$\text{protein precipitated} \rightleftharpoons \text{protein in solution}$$

an approximate ΔH for the reaction can be calculated. By representing the equilibrium constant for the reaction as,

$$Keq = \frac{[sol]}{[ppt]}$$

where the activity of the precipitate is taken as 1.00, ΔH can be found from,

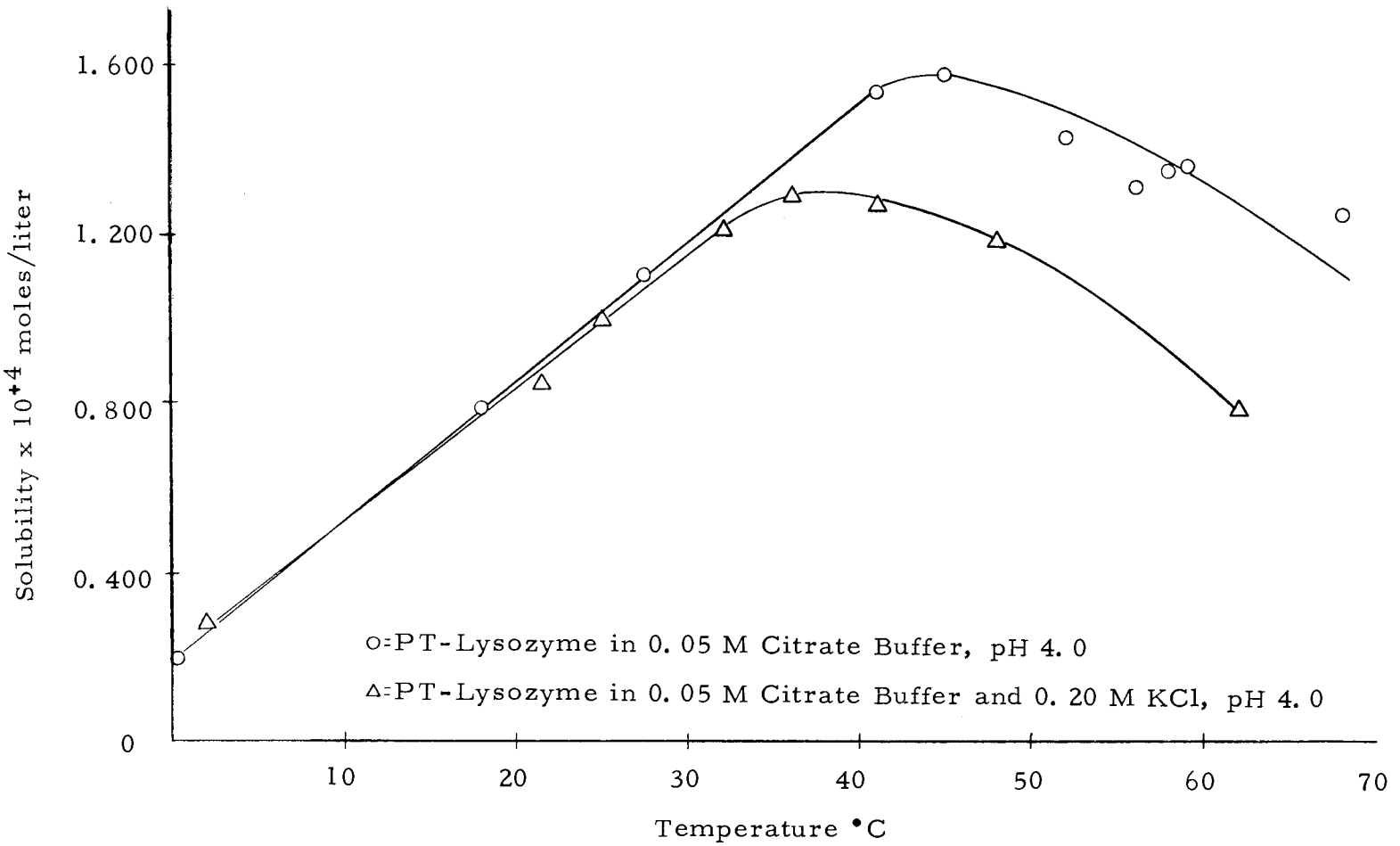


Figure 1. Temperature dependent solubility of PT-Lysozyme.

$$\frac{d(\ln Keq)}{d(1/T)} = \frac{-\Delta H}{R}$$

where T is the absolute temperature and R is the universal gas constant. Then, if ln Keq were plotted against 1/T (Figure 2), the slope of the curve at any point is equal to $-\Delta H/R$.

Table 1. Enthalpy of solution of PT-lysozyme

Temperature (°C)	ΔH(cal/deg. mole)
0°	+14, 000
18°	+ 9, 000
28°	+ 5, 000
41°	+ 1, 000

The range of values for ΔH for PT-lysozyme (Table 1) are of the same order of magnitude as those which have been similarly determined for casein, lactoglobulin, carboxyhemoglobin and serum albumin sulfate (horse) (Cohn and Ferry, 1943). These results would indicate that PT-lysozyme is not an unreasonable model for studying reversible protein aggregations.

Since ΔH is positive from 0° to 41°, and since hydrophobic bonds are more stable at higher temperatures up to about 60° (Kauzmann, 1959, and Scheraga, 1963) the dissolution of PT-lysozyme cannot be regarded as simple disruption of hydrophobic bonds between the added tyrosyl residues.

However, if the aggregation of PT-lysozyme were due

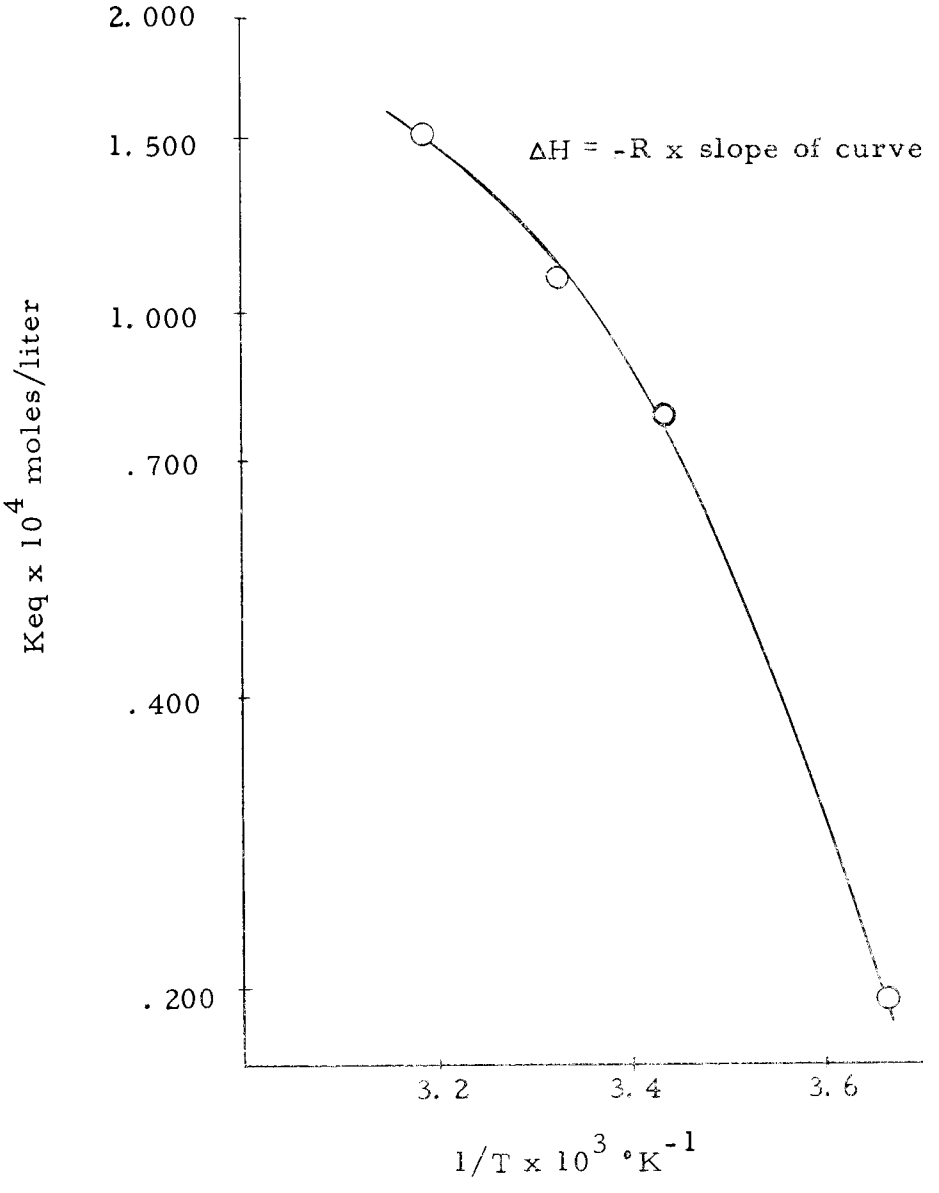


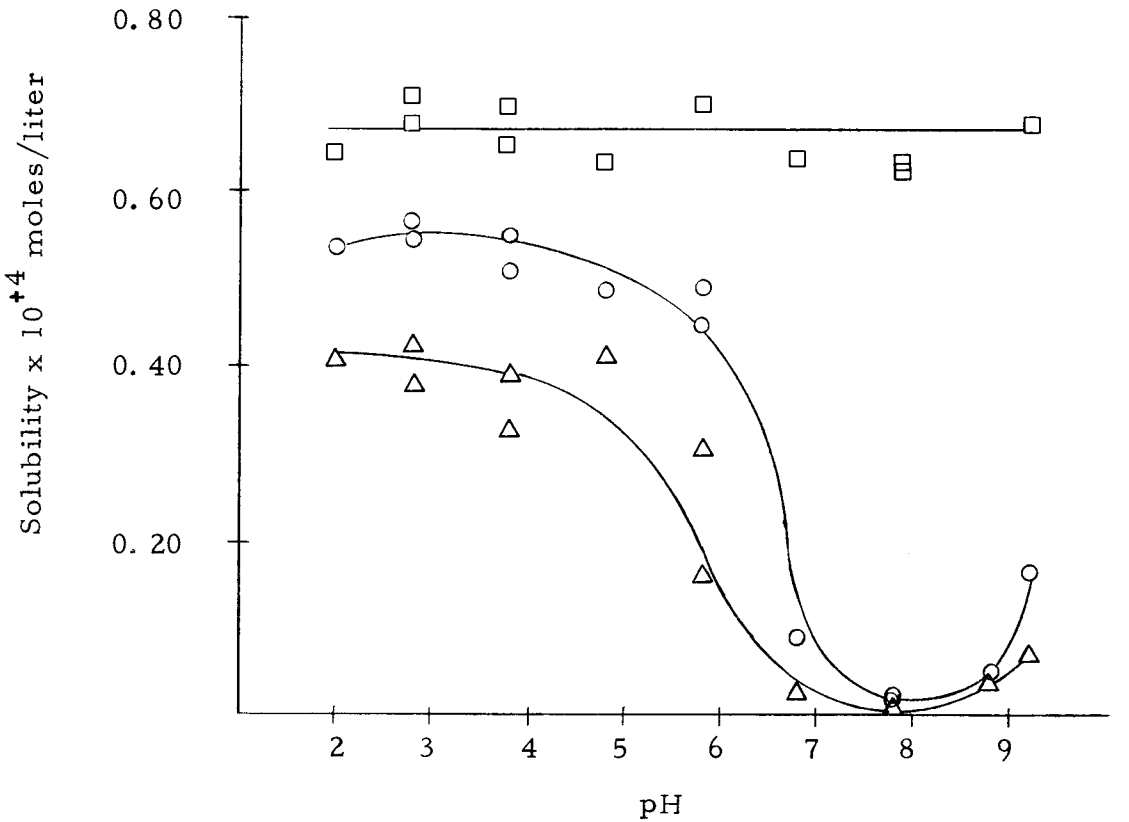
Figure 2. Van 't Hoff plot of log Keq versus inverse of absolute temperature for the temperature dependent solubility of PT-Lysozyme in 0. 05 M citrate buffer, pH 4. 0.

primarily to electrostatic interactions, one would expect that increasing the dielectric constant of the solution would increase the solubility of the protein (Kauzmann, 1959). But, as can be seen in Figure 1, the solubilities are lower at higher ionic strengths, hence we may rule out electrostatic interactions as a predominant factor in PT-lysozyme insolubility.

Preliminary studies of POMT-lysozyme also show a positive enthalpy of solution, further indicating that interactions of charged or polar groups with the phenolic hydrogen of tyrosine cannot wholly be responsible for the PT-lysozyme aggregation.

pH Dependent Solubility of PT-Lysozyme and POMT-Lysozyme

The effect of pH on the solubility of PT-lysozyme and POMT-lysozyme is of special interest not only since lysozyme is soluble under the conditions studied, but also since the insolubility of both lysozyme derivatives falls in the same pH range as the insolubility of polytyrosyl acetamidinated ribonuclease (Lynn, 1966) and polytyrosyl trypsin (Glazer, Bar-Eli and Katchalski, 1962). As can be seen in Figure 3, the solubility of POMT-lysozyme parallels that of PT-lysozyme, having a solubility minimum at pH 8. That this aggregation is due to charge interactions of, or hydrogen bonding to, the phenolic hydrogen of tyrosine is clearly ruled out by the similar insolubility of the O-methyltyrosyl derivative. It also should be



□ = Lysozyme; 0.65 x 10^{-4} moles/liter = 100% solubility
○ = POMT-Lysozyme; 0.60 x 10^{-4} moles/liter = 100% solubility
△ = PT-Lysozyme; 0.42 x 10^{-4} moles/liter = 100% solubility
Ionic strength = 0.10
For buffers used, see Appendix

Figure 3. pH Dependent solubility of PT-lysozyme and POMT-lysozyme.

noted that the isoelectric point of lysozyme is between 10.5 and 11 (Alderton, Ward and Fevold, 1945), and that aggregation at pH 8 cannot be due to elimination of overall electrostatic repulsion. Although it is probable that the modification of lysozyme by adding tyrosyl peptides changes the isoelectric point slightly, as a titratable ϵ - amino group, $pK_a = 10.53$ is exchanged for an α - amino group, $pK_a = 9.11$ (Edsall, 1943), and also, since the pK_a of the tyrosine hydroxyl is 10.07 (Edsall, 1943), it can be seen that the change of the isoelectric point of lysozyme by added tyrosyl residues would be negligible.

Effect of Salts and Denaturing Agents of the Solubility on PT-Lysozyme and POMT-Lysozyme

In order to relate the interactions of the lysozyme derivatives which cause aggregation to those which stabilize most proteins, the effect of a number of salts from the Hofmeister or lyotropic series of ions as well as the effect of guanidine hydrochloride and urea on the solubility of aggregated PT-lysozyme and POMT-lysozyme was studied. The effectiveness of these compounds in denaturing, depolymerizing and dissociating proteins generally follows the same order as their inhibition of a number of enzymes (Robinson and Jencks, 1959b). For example, an increasing order of effectiveness of anions for inhibiting and disrupting β-amylase found by Warren and Cheatum (1966) is $CH_3COO^- < Cl^- < SCN^-$. As shown in

Figures 4 - 8,this same order was found to be increasingly effective in dissociating aggregated PT-lysozyme and POMT-lysozyme. The important point is that urea and guanidine hydrochloride do have a marked effect on the solubility of these lysozyme derivatives.

The interpretation of these results is not clear, especially in view of the different mechanisms proposed for action of urea and guanidine hydrochloride in disrupting protein structure. Kauzmann (1959) suggests that urea disrupts hydrophobic bonds because of its ability to form inclusion complexes with hydrocarbons, while Klotz (1960) argues that this mode of action applies to only straight chain hydrocarbons of six carbon units, and not to aromatic amino acids. Tanford (1964), from a consideration of the free energy of transfer of various amino acids from water to urea or salt solutions, proposes that urea favors dissociation that would expose peptide, amide and/or hydrophobic groups while inorganic salts would favor only dissociation of charged groups and would inhibit dissociation of uncharged groups. In studying the activity coefficient and solubility of acetyltetraglycine ethyl ester, Robinson and Jencks (1965a) have suggested that urea and guanidine hydrochloride could denature proteins by decreasing the activity coefficient of the exposed amide and peptide groups in the denatured protein. The mechanism of action proposed is hydrogen bonding to the polarizable peptide oxygen. Robinson and Jencks (1965b) have further stressed the possibility

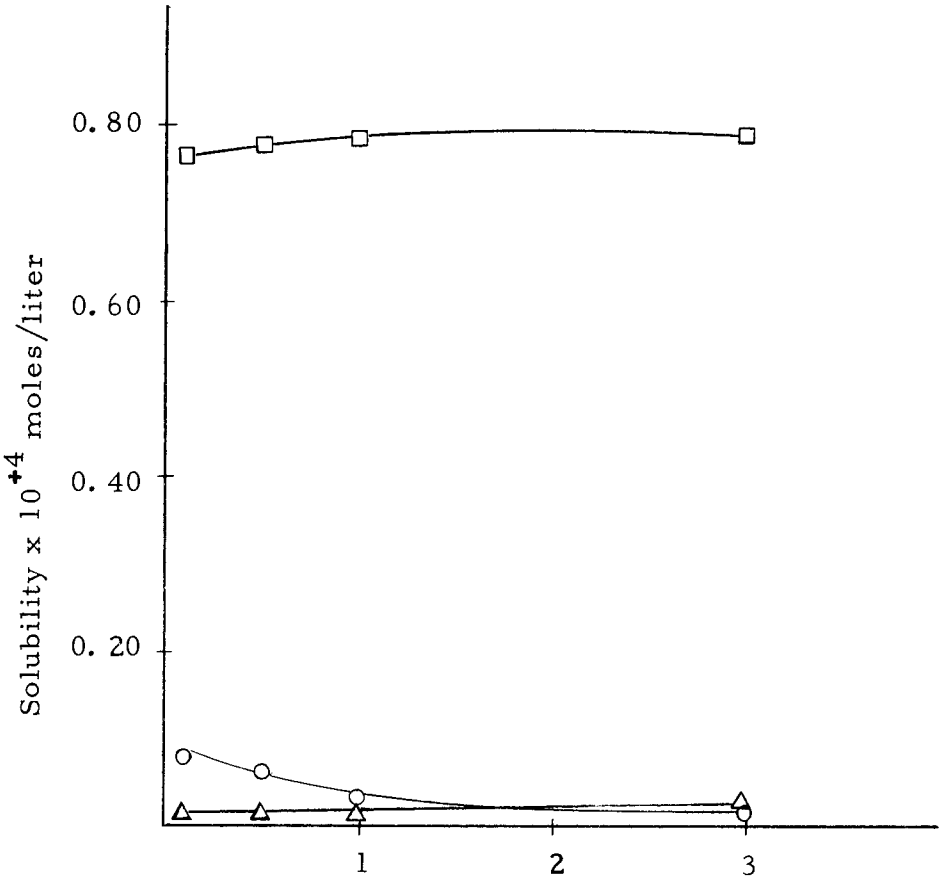


Figure 4. Potassium acetate concentration dependent solubility of PT-lysozyme and POMT-lysozyme.

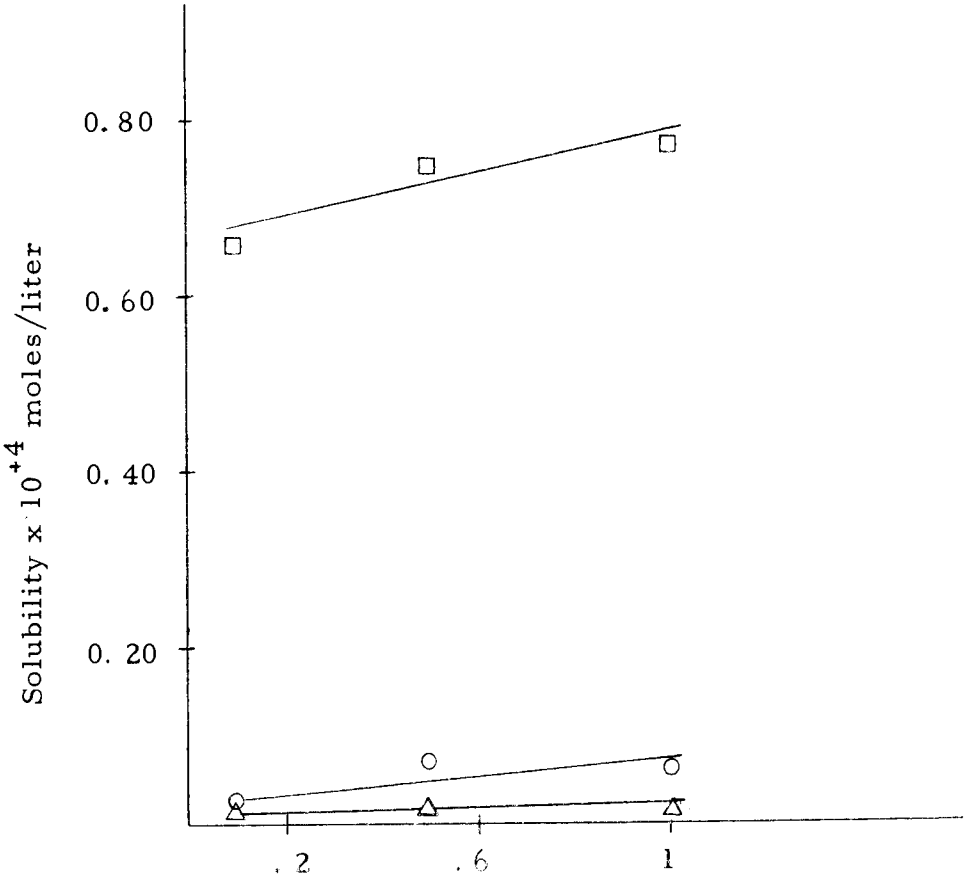
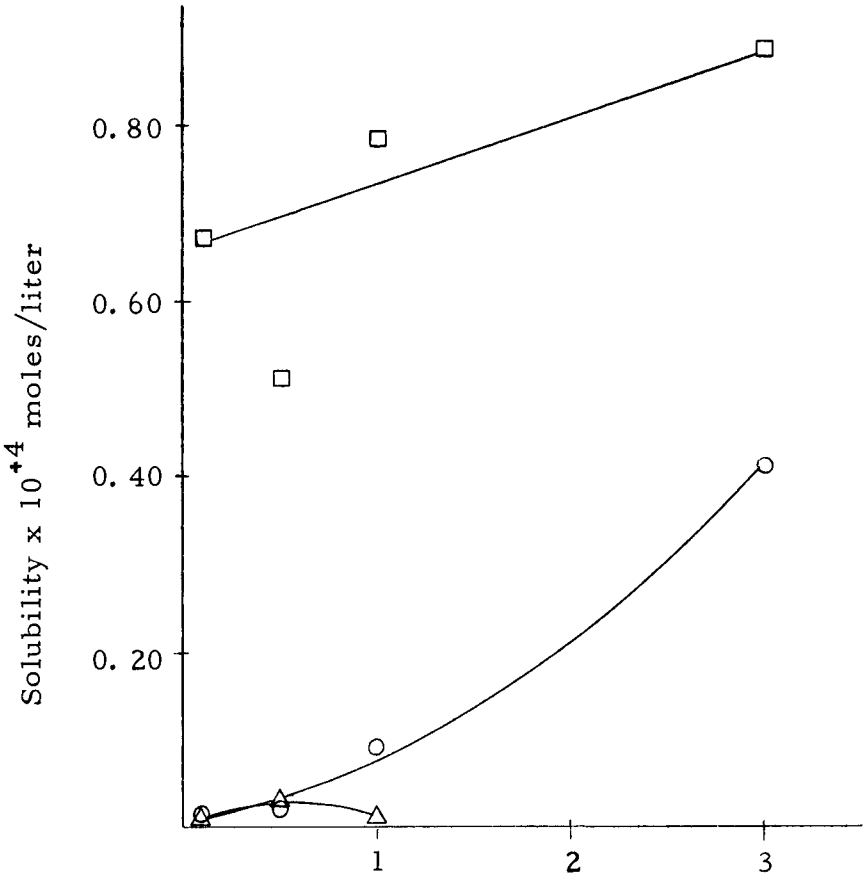


Figure 5. Potassium chloride concentration dependent solubility of PT-lysozyme and POMT-lysozyme.



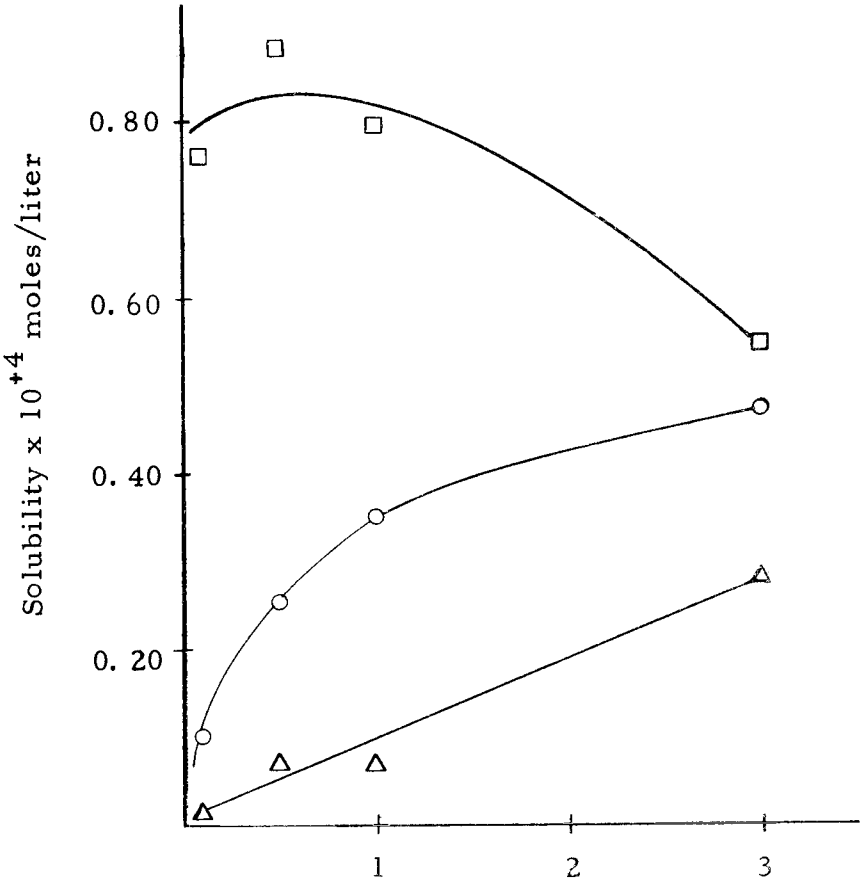
Potassium Thiocyanate Concentration, moles/liter in 0.005 M Tris buffer, pH = 7.2

□ = Lysozyme; 0.65×10^{-4} moles/liter = 100% solubility

○ = POMT-Lysozyme; 0.60×10^{-4} moles/liter = 100% solubility

△ = PT-Lysozyme; 0.42×10^{-4} moles/liter = 100% solubility

Figure 6. Potassium thiocyanate concentration dependent solubility of PT-lysozyme and POMT-lysozyme.



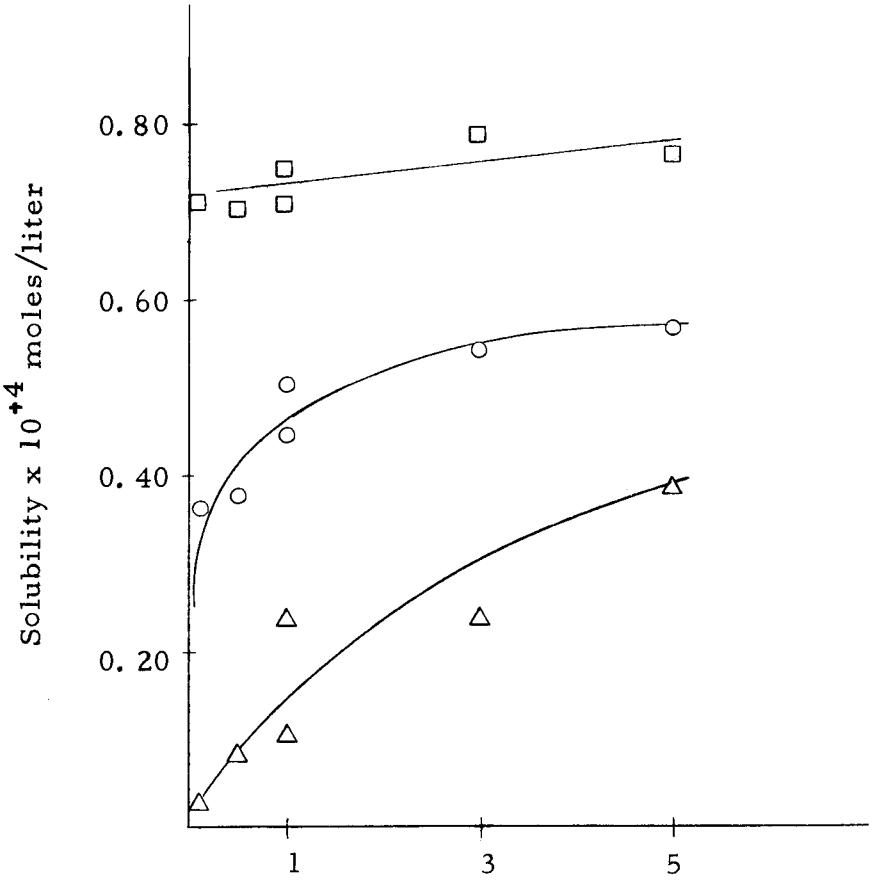
Guanidine Hydrochloride Concentration, moles/liter in 0.005 M Tris buffer pH = 6.9

□ = Lysozyme; 0.65×10^{-4} moles/liter = 100% solubility

○ = POMT-Lysozyme; 0.60×10^{-4} moles/liter = 100% solubility

△ = PT-Lysozyme; 0.42×10^{-4} moles/liter = 100% solubility

Figure 7. Guanidine hydrochloride concentration dependent solubility of PT-lysozyme and POMT-lysozyme.



Urea Concentration, moles/liter in 0.005 M Tris buffer, pH = 6.8

□ = Lysozyme; 0.65×10^{-4} moles/liter = 100% solubility

○ = POMT-Lysozyme; 0.60×10^{-4} moles/liter = 100% solubility

△ = PT-Lysozyme; 0.42×10^{-4} moles/liter = 100% solubility

Figure 8. Urea concentration dependent solubility of PT-lysozyme and POMT-lysozyme

of aromatic-amide interactions, ascribed to the interaction of the polarizable π - electrons of the aromatic ring with the amide or possible weak molecular complexes.

In view of these possible modes of interaction, it would not be unreasonable that urea or guanidine hydrochloride interacted with the added peptide groups as well as with the aromatic rings of tyrosine and O-methyl tyrosine. Hence, the fact that urea dissolves PT-lysozyme and POMT-lysozyme does not imply that their insolubility is due solely to hydrophobic interactions.

These results also demonstrate the greater solubility of POMT-lysozyme compared to that of PT-lysozyme. Whether this solubility difference is due to the lesser degree of modification of POMT-lysozyme or to a difference in the side chains of the amino acids is presently unresolved.

CONCLUSIONS

The general assumption that the different solubility properties of lysozyme derivatives are due to the added groups appears satisfactory since unmodified lysozyme remained soluble under all the conditions. The magnitude of the interactions as indicated by the enthalpy of solution of PT-lysozyme are of the same order of magnitude as other protein aggregations.

Nevertheless, for this model system, a single type of interaction for the aggregation cannot be specified. That hydrophobic interactions are not involved is suggested by the positive enthalpy of solution of these derivatives. However, the inability of an increasing concentration of KCl or CH_3COOK to weaken the aggregation of these derivatives suggests that an ionic type interaction is not entirely responsible. The parallel solubilities of PT-lysozyme and POMT-lysozyme over a pH range eliminates the importance of the phenolic hydrogen as a hydrogen bond donor. This is not to say that tyrosyl residues cannot act as hydrogen bond donors in other systems, for it has been demonstrated by Li, Riehm and Scheraga (1966) that tyrosyl carboxylate interactions are important in stabilizing the structure of ribonuclease. In this laboratory, preliminary results show that succinylated lysozyme aggregates with both PT-lysozyme and POMT-lysozyme at about pH 5 under conditions at which all

derivatives separately are soluble (Tiffany, 1966). Such results would imply that the polarizable aromatic rings and/or the amide groups may be interacting. In addition, the side chains of both tyrosine and O-methyltyrosine can act equally well as hydrogen bond acceptors, as demonstrated by the pK_a's for protonated anisole and phenol. For $[C_6H_5OHCH_3]^+$, $pK_a = -6.54$; while for $[C_6H_5OH_2]^+$, $pK_a = -6.74$ (Arnett and Wu, 1960).

Since a number of interactions of the added polypeptides on lysozyme may participate in aggregation, perhaps the importance of this study lies in demonstrating the ability of tyrosyl peptides to undergo more than just hydrophobic interactions and interactions as hydrogen bond donors. In view of the abnormal pK_a, optical rotatory dispersion and ultraviolet spectra of polytyrosine compared to the monomer (Fasman, Bodenheimer and Lindblow, 1964) a specific tyrosyl-tyrosyl interaction may exist which cannot be delineated as singly hydrophobic, ionic or polar interaction.

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APPENDIX

Observed Optical Densities at 282 mμ After Final Dilution for Figures 1, 3, 4, 5, 6, 7 and 8

Figure 1

0.05 M Citrate Buffer pH 4.0		0.05 M Citrate Buffer 0.20 M KCl, pH 4.0	
Temperature, °C	O. D.	Temperature, °C	O. D.
0	.035	2	.050
18	.140	22	.150
28	.195	25	.178
41	.271	32	.215
45	.280	36	.228
52	.252	41	.225
56	.231	48	.210
58	.239	62	.140
59	.240		
68	.221		

Figure 3

Buffer	pH	Lysozyme O. D.	PT-Lysozyme O. D.	POMT-Lysozyme O. D.
KCl-HCl	2.0	.212	.208	.237
KCl-HCl	2.8	.231	.215	.238
Formate	2.8	.222	.194	.272
Formate	3.8	.214	.168	.223
Acetate	3.8	.228	.198	.243
Acetate	4.8	.207	.212	.215
Acetate	5.8	.230	.157	.217
Phosphate	5.8	.230	.084	.198
Phosphate	6.8	.209	.017	.040
Phosphate	7.8	.207	.004	.006
Tris[§]	7.8	.207	.004	.007
Tris	8.8	----	.021	.023
Tris	9.2	.222	.035	.073

[§]Tris(hydroxymethyl)aminomethane

Figure 4

Concentration of Potassium Acetate in moles/liter	Lysozyme O. D.	PT-Lysozyme O. D.	POMT-Lysozyme O. D.
0. 1	. 250	. 010	. 035
0. 5	. 255	. 011	. 028
1. 0	. 257	. 009	. 016
3. 0	. 259	. 013	. 010

Figure 5

Concentration of Potassium Chloride in moles/liter	Lysozyme O. D.	PT-Lysozyme O. D.	POMT-Lysozyme O. D.
0. 1	. 215	. 007	. 018
0. 5	. 246	. 009	. 031
1. 0	. 252	. 009	. 027

Figure 6

Concentration of Potassium Thiocyante in moles/liter	Lysozyme O. D.	PT-Lysozyme O. D.	POMT-Lysozyme O. D.
0. 1	. 221	. 007	. 008
0. 5	. 180	. 025	. 018
1. 0	. 272	. 020	. 060
3. 0	. 348	ppt.	. 240

Figure 7

Concentration of Guanidine Hydrochloride in moles/liter	Lysozyme O. D.	PT-Lysozyme O. D.	POMT-Lysozyme O. D.
0.1	.250	.010	.045
0.5	.290	.036	.115
1.0	.244	.035	.155
3.0	.181	.143	.210

Figure 8

Concentration of Urea in moles/liter	Lysozyme O. D.	PT-Lysozyme O. D.	POMT-Lysozyme O. D.
0.1	.232	.015	.160
0.5	.230	.043	.165
1.0	.232	.052	.195
1.0	.245	.123	.222
3.0	.258	.123	.239
5.0	.249	.197	.249