Identification of Specific Interactions between Amino Acids[1]

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Abstract: In order to further elucidate the nature of the interactions which determine the tertiary structure of proteins a study was undertaken to identify specific interactions between amino acids. The solubilities of common amino acids were determined in pairwise combinations. L-Arginine hydrochloride was effective in increasing the solubility of L-tryptophan, L-tyrosine, L-phenylalanine, and L-asparagine. L-Histidine hydrochloride was effective in increasing the solubility of L-tryptophan, L-tyrosine, and L-phenylalanine, while L-proline was effective in increasing the solubility of L-tryptophan and L-tyrosine. The solubility data were used to estimate apparent dissociation constants for the following complexes: L-arginine–L-asparagine, 2.6 *M*; L-arginine–L-phenylalanine, 2.2 *M*; L-arginine–L-tryptophan, 0.80 *M*; L-arginine–L-tyrosine, 1.3 *M*; L-histidine–L-tryptophan, 0.61 *M*; L-proline–L-tryptophan, 3.9 *M*; L-proline–L-tyrosine, 7.3 *M*.

There is a considerable amount of experimental evidence which suggests that rather strong and specific interactions between amino acid side chains are important determinants of the shape of protein molecules in solution. For example: (1) Maling and Yanofsky[3] and Yanofsky, *et al.*,[4] obtained mutationally altered A proteins of tryptophan synthetase, and showed that replacement of a single amino acid residue could cause an increase or a decrease in the stability of the A protein toward denaturation by heat or acid.

(2) Yanofsky, *et al.*,[5] showed that certain mutationally altered A proteins which were inactive because of a single amino acid replacement could be mutationally converted to active A proteins by single amino acid replacements 36 amino acid residues from the site of the original replacement.

(3) It is well established that interactions between a small molecule and a large protein molecule can lead to pronounced changes in the conformation of the protein, or stabilization of the original conformation.[6] Because of the limited size of the small molecule, these observations demonstrate that a small number of specific noncovalent bonds can alter the structure of proteins.

Kauzmann[7] has reviewed the evidence which suggests that interactions between apolar residues are important determinants of the tertiary structure of proteins. The exact nature of these apolar or hydrophobic interactions has been the topic of much discussion. Two factors seem to be important in determining the extent of interactions between apolar residues in water.

(1) When an apolar molecule dissolves in water, a shell of water molecules is thought to surround the surface of the apolar molecule. According to Némethy and Scheraga[8] the shell of water molecules is unimolecular. Water molecules in this shell are thought to be more ordered and more involved in hydrogen bonding than the water molecules in the bulk of the medium. The entropy lost by the water molecules in forming the shell is not compensated by the decrease in enthalpy which accompanies the formation of additional hydrogen bonds, so that the participation in shell formation by a large fraction of the water molecules is not feasible. Since the number of water molecules needed to form the shells around apolar residues depends on the surface area of the residues exposed to the water, apolar residues tend to associate into aggregates in order to reduce the area of their surface which is exposed to the water. The theory of Némethy and Scheraga[8] might qualitatively account for specificity in the formation of hydrophobic bonds, since residues would tend to associate when they had large complementary surfaces which could come close together and exclude all the water molecules that would normally form the shell between the two surfaces.

(2) van der Waals forces might also be important in the formation of specific noncovalent bonds. The van der Waals energy of interaction between two atoms falls off with the sixth power of the distance between the two atoms. Watson[9] noted that only groups with complementary geometry could come sufficiently close so that significant van der Waals forces could exist between several atoms in both groups.

Little information is available concerning the identity of any *specific* interactions between amino acid side chains which could be important in defining the tertiary structure of proteins. The authors decided, therefore, to investigate the interactions between the side chains of the free amino acids. This was accomplished by studying the effect of an amino acid on the solubility of various other amino acids in aqueous solutions.

Experimental Section

Materials. Amino acids, NRC A grade, were obtained from Calbiochem and were used without further purification.

Methods. Solubility Determinations. Solutions containing amino acids were stirred continuously for several days, in 13 × 100 mm test tubes sealed with Parafilm, using Cole-Parmer 3 × 10 mm magnetic stirring bars and a Thomas magnetic stirrer. Constant temperature was maintained by placing the test tubes in a jacketed 250-ml beaker which served as a constant-temperature water bath.

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(2) To whom inquiries regarding this work should be directed

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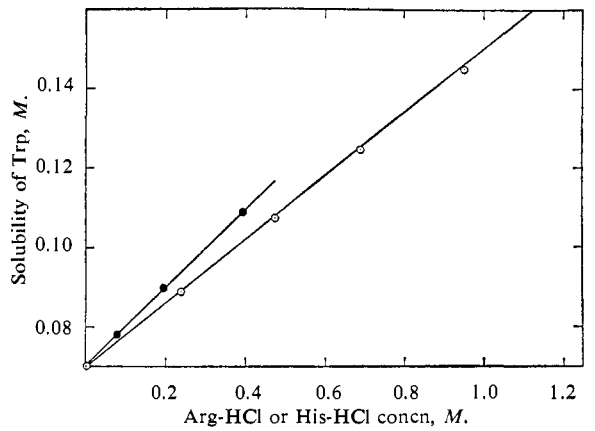

Figure 1. The effect of L-histidine hydrochloride and L-arginine hydrochloride on the solubility of L-tryptophan in water at 25°: L-histidine hydrochloride, ●; L-arginine hydrochloride, ⊙. L-Histidine (7.6% of the concentration of L-histidine hydrochloride) was present in the histidine hydrochloride solutions.

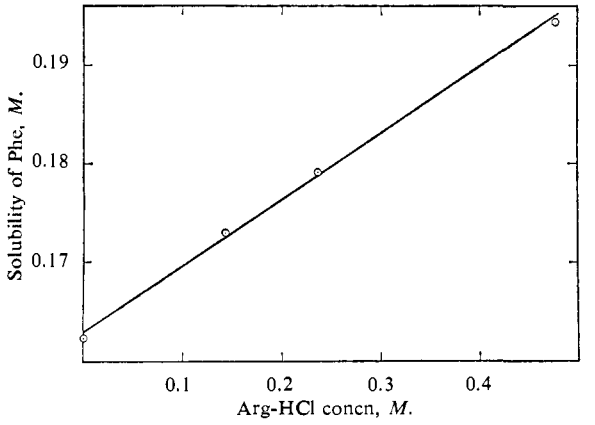

Figure 2. The effect of L-arginine hydrochloride on the solubility of L-phenylalanine in water at 25°.

The water in the beaker was maintained at 25 ± 0.05° by circulating water from a Lauda constant temperature bath through the jacket. Samples of the liquid phase were withdrawn by sucking the solution through a cotton plug inserted in the neck of a disposable Pasteur capillary pipet. An aliquot (0.1–0.5 ml) was removed from the body of the Pasteur capillary pipet with a Lambda ultramicro pipet. The concentrations of amino acids in the liquid phase usually reached a constant value after 2 days of stirring. For the several cases tested, the values obtained for the solubility of the amino acids were independent of the direction of approach to equilibrium. When equilibrium was approached from the supersaturated state, the mixture of amino acids and water was stirred at 60° for several minutes before insertion into the 25° bath. The solubilities used to determine the dissociation constants listed in Table I were evaluated from at least two determinations which were taken 24 hr apart and were within 2% of each other.

The values determined by us for the intrinsic solubility of amino acids in water were usually in good agreement with the previously determined values of Dalton and Schmidt.[10] However, our values for the solubility of tryptophan (0.070 *M*) and phenylalanine (0.161 *M*) are closer to the values reported by Nozaki and Tanford[11] for the solubility of tryptophan (0.068 mol/1000 g of water) and phenylalanine (0.169 mol/1000 g of water) than the values (0.056 mol/1000 g of water and 0.179 mol/1000 g of water) reported by Dalton and Schmidt.[10]

Routinely, the solubilities of amino acids in pairs of two were determined only once, after stirring the solutions for 3–7 days. Several redeterminations of the solubilities of amino acids in a given pair were carried out, when their solubilities in the pair differed significantly from their intrinsic solubilities. The concentrations of methionine, phenylalanine, tryptophan, and tyrosine were usually determined from their ultraviolet spectra in aqueous solution. At the concentration levels used in determining ultraviolet spectra, the presence of the other amino acid had no effect on the ultraviolet spectra. (High concentrations of L-histidine hydrochloride caused a slight decrease in the absorbance of L-tryptophan. A 5% decrease in the molar absorptivity at 275 mμ of L-tryptophan was observed in 0.867 *M* L-histidine hydrochloride–0.08 *M* L-histidine.) The concentrations of other amino acids were determined by the method of Spackman, *et al.*,[12] except after development of the color, the absorbance was measured continuously at 570 mμ on a Gilford Model 2000 multiple sample absorbance recorder using 0.2- and 1-cm flow-through cells. Areas under the peaks were measured with a compensating polar planimeter sold by Gelman Instrument Co. Ultraviolet spectra were determined on a Cary Model 15 recording spectrophotometer.

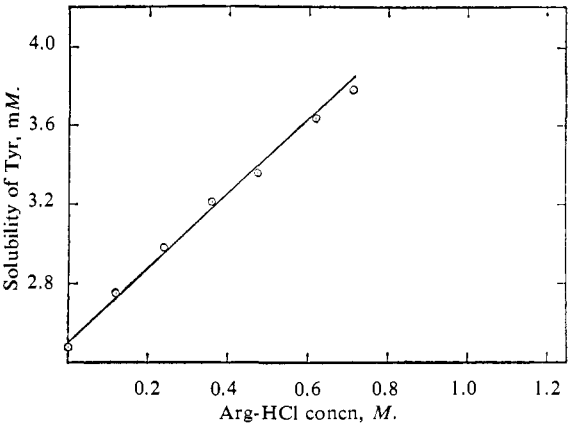

Figure 3. The effect of L-arginine hydrochloride on the solubility of L-tyrosine in water at 25°.

Results

Interactions Involving L-Arginine Hydrochloride and L-Histidine Hydrochloride. For several amino acids,[13] the solubility of the amino acid in water was compared with its solubility in the following solutions: 0.5 *M* arginine hydrochloride, 0.5 *M* histidine hydrochloride, and 0.5 *M* lysine hydrochloride. Arginine hydrochloride and histidine hydrochloride were most effective in increasing the solubility of tryptophan, tyrosine, phenylalanine, and asparagine. No pronounced differences were observed between the solubilities of amino acids in 0.5 *M* lysine hydrochloride and their solubilities in water.

The linear relationships illustrated in Figures 1–3 indicate that the increase in solubility of tryptophan, tyrosine, and phenylalanine in solutions of arginine hydrochloride could be rationalized by assuming that a 1:1 complex forms between arginine and the aromatic amino acids.

If two compounds, A and B, form a 1:1 complex with a dissociation constant of K_D, it can be shown that when the solid phase consists solely of compound B, eq 1

$$[B^0] = \frac{K_S[A^0]}{K_S + K_D} + K_S \qquad (1)$$

holds. The solubility of B in pure water is given by

(10) (a) J. B. Dalton and C. L. A. Schmidt, *J. Biol. Chem.*, **103**, 549 (1933); (b) J. B. Dalton and C. L. A. Schmidt, *ibid.*, **109**, 241 (1935); (c) J. B. Dalton and C. L. A. Schmidt, *J. Gen. Physiol.*, **19**, 767 (1936).

(11) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963).

(12) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(13) Namely, Ala, Asn, Gln, Gly, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr, and Val.

K_S, and $[A^0]$ and $[B^0]$ represent the total concentrations of the two compounds in the liquid phase.

Figure 1 also suggests that tryptophan forms a 1:1 complex with histidine. The increase in solubility of asparagine in solutions of arginine hydrochloride is also consistent with the formation of a 1:1 asparagine–arginine complex. Apparent dissociation constants for these 1:1 complexes are listed in Table I. As shown in Table I the apparent dissociation constant for the complex of L-arginine and L-tryptophan does not differ significantly from the dissociation constant for the complex L-arginine–D-tryptophan.

Table I. Apparent Dissociation Constants for 1:1 Complexes of Amino Acids at 25° [a]

Complex	K_D, M	Complex	K_D, M
L-Arg–L-Asn	2.6[b]	L-Arg–L-Tyr	1.3[b]
L-Arg–L-Phe	2.2[b]	L-His–L-Trp	0.61[b]
L-Arg–L-Trp	0.80[b]	L-Pro–L-Trp	3.9
L-Arg–D-Trp	0.76[b]	L-Pro–L-Tyr	7.3

[a] Estimated accuracy, ±5%. [b] To calculate K_D, the forms of His and Arg with a net positive charge of one were assumed to form the complex.

It should be noted that at concentrations of histidine hydrochloride and arginine hydrochloride higher than shown in Figures 1–3, negative deviations from eq 1 are observed. Precipitation of the amino acid complex and nonspecific salt effects are probably responsible for these deviations.

Although histidine hydrochloride markedly increases the solubility of phenylalanine and tyrosine,[14] negative deviations from eq 1 were observed at low concentrations of histidine hydrochloride. Further work is required to characterize these interactions.

Interactions Involving L-Proline. Proline was found to be most effective in increasing the solubility of tryptophan and tyrosine. The increase in solubility of tryptophan and tyrosine in proline solutions was interpreted in terms of eq 1. As shown in Figure 4 positive deviations from eq 1 were observed for the solubilities of tryptophan in proline solutions. This result could be explained by assuming the complex $(proline)_2$–tryptophan forms at high proline concentrations. Apparent dissociation constants estimated for the 1:1 complexes of proline are also listed in Table I. In comparison to proline, alanine, glycine, serine, and threonine were not effective in increasing the solubility of tyrosine and tryptophan. Proline (0.7 M) did not increase the solubility of isoleucine, leucine, methionine, phenylalanine, and valine. In fact these amino acids were slightly less soluble (2–15%) in 0.7 M proline than in water.

Other Interactions between Neutral Amino Acids. Additional solubility studies were undertaken in an attempt to identify other pairs of interacting L-amino acids. The solubilities of alanine, asparagine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tyrosine, tryptophan, and valine were determined in pairwise combinations. Saturated solutions of the more soluble amino acids, glycine, alanine, serine, and threonine, altered the solubility of other amino acids. At concentrations of 0.7 M the effects of these soluble amino acids on the solubility of the other amino acids were small and could not be ascribed to specific interactions.

(14) The solubility of phenylalanine increases from 0.16 M in water to 0.21 M in 0.5 M histidine hydrochloride. The solubility of tyrosine increases from 2.5×10^{-3} M in water to 3.8×10^{-3} M in 0.5 M histidine hydrochloride.

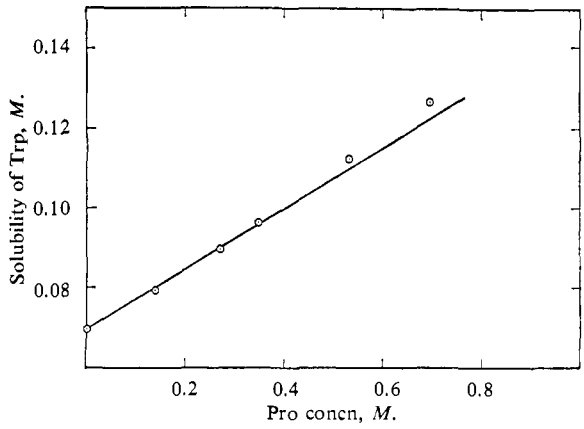

Figure 4. The effect of L-proline on the solubility of L-tryptophan in water at 25°.

The following pairs of amino acids were found to form mixed solid phases of low solubility:[15] glutamine–phenylalanine, glutamine–leucine, isoleucine–leucine, leucine–methionine, isoleucine–methionine, and phenylalanine–methionine. The amino acids in the liquid phase were in equilibrium with the amino acids in the mixed solid phases at concentrations below their intrinsic solubilities in water. It is unusual for organic compounds to mix in the solid phase, and it is interesting that these pairs of amino acids form mixed solid phases. Further work is required to demonstrate the existence of any specific interactions between these amino acids in solution.

Discussion

When the ability of one amino acid to alter the solubility of another amino acid depends on the nature of the side chains of the two amino acids, it is unlikely that electrostatic interactions between ammonium and carboxylate groups are solely responsible for these changes in solubility. Cohn[16] and his coworkers have characterized electrostatic interactions between dipolar ions. In their studies of the solubility of cystine and asparagine in the presence of some other amino acids and peptides, they observed that amino acids with apolar side chains were not as effective as glycine in solubilizing asparagine and cystine. The change in free energy of interacting dipolar ions without apolar side chains could be accounted for by applying electrostatic theory to various theoretical models for interacting dipolar ions. The contributions from apolar side chains to the interactions between amino acids reported here cannot be explained by the theories for electrostatic interactions discussed by Cohn. The failure of lysine hydrochloride to mimic the solubilizing effects of arginine hydrochloride and histidine hydrochloride is also

(15) Further work is required to classify these mixed solid phases as solid solutions or solid compounds.

(16) Cohn has discussed the results of several studies dealing with electrostatic interactions involving dipolar ions in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp 217–275.

difficult to rationalize in terms of theoretical models based on electrostatic theory.

The specific solubilizing effects observed for arginine hydrochloride, histidine hydrochloride, and proline reflect the ability of these amino acids to specifically decrease the activity coefficients of certain other amino acids. These changes in activity coefficients must reflect specific interactions between pairs of amino acids, since the observed changes in activity coefficients cannot be explained in terms of nonspecific medium effects. It is realized that the change in activity coefficient of one amino acid in the presence of another amino acid is complicated by ionic as well as apolar interactions. Therefore, the linear dependence of the solubility of one amino acid on the concentration of another amino acid should only be taken as circumstantial evidence for the formation of a 1:1 complex. Since the apparent value for the dissociation constant estimated from solubility data reflects the strength of the interaction even if no complex actually forms,[17] we have chosen to use this parameter to characterize each interaction.

It is not surprising that histidine hydrochloride and arginine hydrochloride appear to interact readily with several other amino acids, since imidazole hydrochloride and guanidine hydrochloride are effective denaturing agents of proteins.[18] Perhaps externally added guanidine hydrochloride or imidazole hydrochloride disrupts specific interactions between amino acid side chains by binding directly to one or more of the interacting side chains.

Robinson and Jencks[19] explain the increased solubility of acetyltetraglycine ethyl ester in guanidine hydrochloride solutions by assuming a 1:1 complex forms between guanidine hydrochloride and acetyltetraglycine ethyl ester. The apparent dissociation constant of this complex is 1.1 *M* and is similar to the values we report for the complexes of arginine hydrochloride. It should be noted, however, that polyfunctional hydrogen bonding is probably responsible for these interactions with guanidine hydrochloride, whereas the interactions listed in Table I cannot be attributed entirely to hydrogen bonding. Arginine hydrochloride is most effective in increasing the solubility of tryptophan, tyrosine, phenylalanine, and asparagine. Nozaki and Tanford[11] studied the increase in solubility of 11 amino acids in urea solution. Their results indicate that urea is also most effective in increasing the solubility of tryptophan, tyrosine, phenylalanine, and asparagine.

Although the apparent dissociation constants listed for the complexes in Table I are large these interactions might be significant in determining the tertiary structure of proteins. Two amino acid side chains forming a complex with a dissociation constant of 2 *M* would be associated about 90% of the time when on the same protein molecule, assuming that on a protein molecule the effective concentration of one residue with respect to another residue is 20 *M*.

Further studies of the colligative properties of solutions of pairs of amino acids and peptides are under way in order to further assess the possible contributions of specific interactions between amino acid side chains to the tertiary structure of proteins.

(17) The free energy change (ΔF_{tr}) for transferring amino acid B from pure water to another medium at the same molar concentration of amino acid B is simply $-RT \ln([B^0]/K_S)$, where K_S and $[B^0]$ represent the molar solubility of amino acid B in water and the other medium respectively. Since the solubility data are represented by eq 1, even if no complex actually forms, $\Delta F_{tr} = -RT \ln[([A^0]/K_S + K_D) + 1]$. When $K_D \gg K_S$ this free energy change may be approximated by $-RT \ln[([A^0]/K_D) + 1]$.

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(19) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2462 (1965).

Communications to the Editor

The Structure of a C_{19}-Diketone Derived from Ryanodine[1]

Sir:

Ryanodine, $C_{25}H_{35}O_9N$, isolated from *Ryania speciosa* Vahl,[2] is of interest because of its high oxygenation and the lack of structural analogies among natural products as well as its extreme and exceptional toxicity[2a,3] and insecticidal action.[2a,4] Hydrolysis leads to pyrrole-α-carboxylic acid and ryanodol, $C_{20}H_{32}O_8$.[2] Structural assignments for ryanodine and ryanodol have been based in part on acid-rearranged products and C_4–C_8 fragments resulting from the action of alkali on these products.[5]

We have degraded ryanodol to a C_{19} compound to which a unique structural assignment can be made. Ryanodol,[6,7] mp 247°, $[\alpha]$ +18°, with excess periodate[8]

(1) This work was sponsored by the U. S. Army Chemical Corps, the U. S. Army Research Office, Durham, and the National Institutes of Health, U. S. Public Health Service. It was presented in part at the Tenth Organic Chemistry Conference, U. S. Army Natick Laboratories, Oct 4, 1966.

(2) (a) E. F. Rogers, F. R. Koniuszy, J. Shavel, Jr., and K. Folkers, *J. Am. Chem. Soc.*, **70**, 3086 (1948); (b) R. B. Kelly, D. J. Whittingham, and K. Wiesner, *Chem. Ind.* (London), 857 (1952), (c) we are greatly indebted to Dr. E. F. Rogers (Merck Sharp and Dohme) for information on his preliminary investigations and for various samples.

(3) W. L. Haslett and D. J. Jenden, *J. Cellular Comp. Physiol.*, **57**, 123 (1961).

(4) K. D. Arbuthnot, *J. Econ. Entomol.*, **51**, 562 (1958).

(5) See K. Wiesner, Z. Valenta, and J. A. Findlay, *Tetrahedron Letters*, 221 (1967), for the most recent structural postulate and references to previous proposals. Consideration of these structures and of our data will be made in a future publication. Also, after this paper was submitted for publication, a structure for ryanodol *p*-bromobenzyl ether based on X-ray diffraction studies appeared by S. N. Srivastava and M. Przybylska, *Can. J. Chem.*, **46**, 795 (1968).

(6) The assistance of Dr. K. Folkers and Merck Sharp and Dohme in securing starting material was of inestimable value.

(7) Satisfactory elementary analyses were obtained for all compounds reported; ultraviolet spectra were taken in ethanol and are reported in $m\mu$ (ϵ); infrared spectra as KBr wafers are reported in cm^{-1}