Thermodynamics of the Denaturation of Lysozyme by Guanidine Hydrochloride. II. Dependence on Denaturant Concentration at 25°*

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ABSTRACT: The fact that guanidine hydrochloride shifts the equilibrium between native and denatured lysozyme toward the denatured form requires that more guanidinium and/or chloride ions are bound to the denatured form than to the native form; or that more water is bound to the native form than to the denatured form; or a combination of these effects. Various hypothetical equilibria are proposed, and it is found that all of them are consistent with the experimental data, so that no definite conclusions concerning the mode of action of guanidine hydrochloride can be reached. All of the hypothetical mechanisms, however, lead to the conclusion that the free energy of stabilization of native lysozyme, relative to the denatured state, under physiological conditions, in the absence of denaturant, is only 10–20 kcal/mole.

It has been shown in the preceding paper (Aune and Tanford, 1969) that the denaturation of lysozyme by GuHCl, at 25° and over a wide range of pH, is a two-state process: only native (N) and fully denatured (D) states contribute significantly to the equilibrium properties. The state of equilibrium can be completely described in terms of a single equilibrium constant K_D, for the reaction $N \rightleftarrows D$. It was shown in addition that there are theoretical reasons for believing that K_D itself can be considered a product of two independent functions (at 25°), one depending upon pH alone and one depending on the activity of GuHCl alone, *i.e.*, we may write

$$K_D = K_D^0 F(a_H) f(a_{GuHCl}) \quad (1)$$

where K_D^0 is a constant that formally represents the value of K_D under conditions where $F(a_H)$ and $f(a_{GuHCl})$ are both equal to unity. The form used for $F(a_H)$ was such that it would become unity at very low pH. The form to be used for $f(a_{GuHCl})$ in this paper will give a value of unity when $a_{GuHCl} = 0$. The validity of eq 1, within experimental error, and within the range of conditions investigated (pH 1.5–5.5, GuHCl concentration 1.5–4 M), was confirmed by the results shown in Figure 7 of the preceding paper. A single expression for $F(a_H)$ was obtained which resulted in values for $K_D/F(a_H)$ that fall on a smooth curve when plotted as a function of the concentration of GuHCl, regardless of the pH of measurement.

It is the purpose of this paper to analyze the dependence of K_D upon the concentration of GuHCl, *i.e.*, to obtain an expression for $f(a_{GuHCl})$, and to consider what information it can provide as to the mode of action of GuHCl as a denaturant.

Formal Representation of $f(a_{GuHCl})$. To obtain a suitable analytic function for $f(a_{GuHCl})$, we first need to know the activity of GuHCl as a function of its molar concentration. Isopiestic vapor pressure measurements from which this information can be obtained have been made by E. P. K. Hade, Jr., in this laboratory. We have found that his results may be represented by the polynomial

$$\log a = -0.5191 + 1.4839 \log C - 0.2562 (\log C)^2 + 0.5884 (\log C)^3 \quad (2)$$

where a designates activity and C the molar concentration. Using this relation, the data of Figure 7 of the preceding paper have been replotted as a function of a_{GuHCl} in Figure 1.

As has been shown elsewhere (Tanford, 1969), the slope of Figure 1, at any given value of a_{GuHCl}, can be rigorously represented in terms of the *preferential* "binding" of the denaturant to the protein, *i.e.*

$$\left(\frac{\partial \ln K_D}{\partial \ln a_{GuHCl}}\right)_{T,pH} = \left(\frac{\partial \ln f(a_{GuHCl})}{\partial \ln a_{GuHCl}}\right)_T = \Delta\bar{\nu}_{GuHCl,pref} \quad (3)$$

where

$$\Delta\bar{\nu}_{GuHCl,pref} = \Delta\bar{\nu}_{GuHCl} - \frac{m_{GuHCl}}{55.5}\Delta\bar{\nu}_W \quad (4)$$

In eq 4, m_{GuHCl} is the molal concentration of GuHCl in the solution, $\Delta\bar{\nu}_{GuHCl}$ represents the difference between the number of moles of GuHCl "bound" to 1 mole of D and N, respectively, at any given value of a_{GuHCl}

$$\Delta\bar{\nu}_{GuHCl} = \bar{\nu}_{GuHCl,D} - \bar{\nu}_{GuHCl,N} \quad (5)$$

while $\Delta\bar{\nu}_W$ is a similar expression for "bound" water molecules

$$\Delta\bar{\nu}_W = \bar{\nu}_{W,D} - \bar{\nu}_{W,N} \quad (6)$$

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It should be observed that the reason for the appearance of both $\Delta\bar{\nu}_{GuHCl}$ and $\Delta\bar{\nu}_W$ in the expression for $\partial \ln K_D/\partial \ln a_{GuHCl}$ lies in the fact that the addition of GuHCl to an aqueous protein solution alters the activity of water as well as that of the denaturant. Effects resulting from this addition cannot therefore be uniquely ascribed to the changing activity of GuHCl. The label "preferential" is affixed to the "binding" defined by eq 4 because $\bar{\nu}_{GuHCl,pref}$ in fact represents the number of GuHCl molecules "bound" to the protein in excess of "binding" of both GuHCl and water in the same ratio in which these components are present in the solvent alone.

The term "bound" has been placed in quotation marks in the preceding discussion because it includes all molecules of water or GuHCl that may be considered as formally associated with the protein molecule, and is not limited to specific site binding. One rigorous definition is that $\Delta\bar{\nu}_{GuHCl}$ and $\Delta\bar{\nu}_W$ represent the number of moles of GuHCl or water that must cross a membrane, separating protein solution from solvent solution (at fixed a_{GuHCl}) in equilibrium with it, for each mole of reaction N → D.

Equations 3–5 apply as written only if the neutral GuHCl molecule is "bound" to the protein as a single entity, and they become ambiguous when cognizance is taken of the fact that GuHCl exists in solution as GuH^+ and Cl^- ions, which can react independently. Actually the equations remain valid provided that $\Delta\bar{\nu}$ is defined in terms of the number of moles of GuHCl or water passing across a membrane, as was done in the previous paragraph, for electrical neutrality requires that net transfer across a membrane must be expressible in terms of neutral components even if only one ion is "bound" to the protein.

It is preferable however, to express the equations in terms of the "binding" of ions *per se*. This may be done by retaining eq 3, but replacing eq 4 by

$$\Delta\bar{\nu}_{GuHCl,pref} = \frac{1}{2}(1 + x)\Delta\bar{\nu}_{GuH^+} + \frac{1}{2}(1 - x)\Delta\bar{\nu}_{Cl^-} - \frac{m_{GuHCl}}{55.5}\Delta\bar{\nu}_W \quad (7)$$

$$x = \frac{1}{2}\mathrm{d} \ln (\gamma_+/\gamma_-)/\mathrm{d} \ln m_{GuHCl} \quad (8)$$

γ_+ and γ_- representing activity coefficients of the constituent ions on the molality scale. (They are generally unknown quantities, and x must normally be taken as zero.) An obvious aspect of eq 7 is that it is not possible to distinguish between the "binding" of GuH^+ and Cl^- ions unless experiments are carried out in which the activities of these ions are measured independently. In an experiment in which the only parameter that is being varied is the number of moles of GuHCl added to the system, equivalent effects are obtained upon the "binding" of either ion as a primary event.[1]

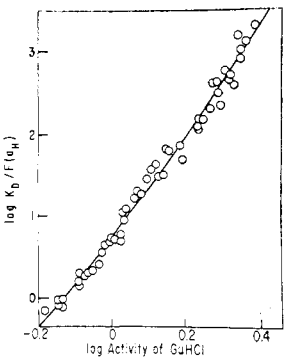

FIGURE 1: A plot of log $[K_D{}^0 f(a_{GuHCl})]$ as a function of the activity of GuHCl. The line is a theoretical one, representing either eq 11 or 15, with the parameters given in the text.

The slope of Figure 1 varies from about 4.5 to about 8 between the lower and upper ranges of a_{GuHCl} encompassed by the figure. This means that the difference in preferential "binding" between native and denatured protein is relatively small, *i.e.*, about 16 GuH^+ plus Cl^- ions at the highest GuHCl concentration.

Simple Models. One wishes to identify the sites of action of the denaturing agent. This involves designing reasonable models that will account for the observed values of $\Delta\bar{\nu}_{GuHCl,pref}$. The simplest possible model is to assume that the denaturing action is due entirely to the binding[2] of GuHCl, and that water binding can be neglected. We first take the ligand to be a neutral GuHCl molecule, which is not very realistic, but which can be treated mathematically in an unambiguous way since activities of GuHCl are known.

We shall assume that binding sites are for single molecules of GuHCl, and that they are noninteracting: at each site there is an equilibrium

$$\text{site} + \text{GuHCl} \rightleftharpoons \text{site (GuHCl)} \quad (9)$$

with an equilibrium constant $k_{i,N}$ for sites on the native proteins, or $k_{i,D}$ for sites on the denatured protein. In analogy

[1] In principle, similar considerations apply to the effect of pH on K_D, at constant activity of GuHCl, as described in the preceding paper. This effect occurs, however, at very low concentrations of the component containing the H^+ ion (*e.g.*, HCl), so that, in the equation corresponding to eq 7 the ratio $m_{HCl}/55.5$ is negligibly small. In considering the effect of pH it is also possible to ascribe the effect uniquely to H^+ ions because the activity of H^+ ions is measured independently, and, though this activity cannot be altered experimentally without simultaneous alteration in the activity of some other ion (*e.g.*, Cl^-), it is easy to demonstrate that the activity of such other ions is not responsible for the observed effects (*e.g.*, by showing that addition of NaCl, KCl, etc., do not produce these effects).

[2] The quotation marks for the word "binding" are omitted from here on, as the simple models invoked here consider the interaction of GuHCl with protein in terms of stoichiometric site binding. It is entirely possible that all or some of the interaction between GuHCl and the protein is in the form of *domain binding*. In this kind of binding a "site" consists of a relatively large volume which can contain water molecules and GuH^+ and Cl^- ions in various proportions. The average composition would have to be described as a more complex function of the activity of GuHCl than any that we have actually employed. Since the simple functions used for $f(a_{GuHCl})$ in the following equations adequately account for the data, a more complex function with more variable parameters would also do so, and nothing is gained by writing an explicit expression based on the concept of domain binding.

with eq 8–10 of the preceding paper (Aune and Tanford, 1969), we would then have

$$f(a_{GuHCl}) = \frac{\prod_{i,D}(1 + k_{i,D}a_{GuHCl})}{\prod_{i,N}(1 + k_{i,N}a_{GuHCl})} \quad (10)$$

each product extending overall binding sites in the two forms of the protein.

The number of parameters of eq 10 that can be determined from the data of Figure 1 is very small and simplifying assumptions clearly need to be made. The binding constants $k_{i,D}$ and $k_{i,N}$ turn out quite small regardless of what assumptions are made, and we have considered that the most reasonable simplification of eq 10 is to assume that the difference between native and denatured states lies in the *number* of binding sites, *i.e.*, that the denatured protein has sites that arise from portions of the molecule which are buried in the interior of the native structure and are therefore absent when the protein is in its native state. With a single average binding constant, k, for all sites, we obtain

$$K_D/F(a_H) = K_D^0 f(a_{GuHCl}) = K_D^0(1 + ka_{GuHCl})^{\Delta n} \quad (11)$$

where $\Delta n = n_D - n_N$ is the difference between the number of sites on the denatured and native protein molecules.

A computer program was designed to give a least-squares fit of the data of Figure 1 to eq 11. The result obtained was $\Delta n = 7.84$, $k = 3.00$, and log $K_D^0 = -4.00$. The curve in Figure 1 is drawn according to eq 11 with these numerical values for the parameters. The nonintegral value for Δn is permissible in view of the approximation of constant k in eq 11. In reality there must be binding sites (if there *are* binding sites for GuHCl at all) with a range of k values, and the equation presumably simulates weak binding sites as fractional sites. The result may be interpreted as indicating that binding sites with $k \simeq 3$ may account for $\Delta n = 6$ or 7, and that the remainder of $f(a_{GuHCl})$ represents the effects of weaker sites.

New information could not be obtained by modification of eq 11 to allow specifically for weaker binding sites. If we were to replace the factor $(1 + ka_{GuHCl})^{\Delta n}$ by a product of two factors $(1 + k_1a_{GuHCl})^{\Delta n_1}(1 + k_2a_{GuHCl})^{\Delta n_2}$, we would have four parameters to determine (apart from K_D^0) instead of two, and the data are not good enough to obtain significant values for them.

The use of eq 9 to describe the binding equilibrium implies that the sites involved are specific for the neutral GuHCl molecule, and that they cannot bind water. The "free" site in such a situation would have the space near the site filled with mobile solvent molecules or ions without preference for a particular kind. An alternative possibility would be that the sites in question can specifically bind either water or GuHCl, so that the equilibrium is a competitive one. For example, GuHCl might compete for the site with a pair of water molecules. In that case the equilibrium might be governed by the process

$$\text{site } (H_2O)_2 + GuHCl \rightleftharpoons \text{site } (GuHCl) + 2\ H_2O \quad (12)$$

and, if k' is the equilibrium constant for this reaction, eq 11 would be replaced by

$$K_D/F(a_H) = K_D^0(1 + k'a_{GuHCl}/a_W^2)^{\Delta n} \quad (13)$$

where a_W is the activity of water.[3] (In using eq 13 we of course automatically ascribe some of the preferential binding of GuHCl to the $\Delta\bar{\nu}_W$ term of eq 4.)

Application of our computer program to eq 13 and the results of Figure 1 gave the following best values for the parameters of the equation: $\Delta n = 6.28$, $k' = 5.1$, and log $K_D^0 = -4.55$. The same qualifications apply to these parameters as were discussed in connection with eq 11.

Because GuHCl is a strong electrolyte, it is much more likely that its ions bind independently to proteins than that they act in concerted fashion to give molecular binding as implied by eq 9 and 12. If ion binding is invoked, the simplest possible equation (assuming that no important changes in hydration accompany denaturation), analogous to eq 11, would be

$$K_D/F(a_H) = K_D^0(1 + k_1a_{GuH^+})^{\Delta n_1}(1 + k_2a_{Cl^-})^{\Delta n_2} \quad (14)$$

where subscripts 1 and 2 refer to binding sites for GuH^+ and Cl^-, respectively. This equation again contains more parameters than can be obtained from the experimental results. In addition, it suffers from the complication that values for a_{GuH^+} and a_{Cl^-} are not available, and, indeed, cannot be obtained without nonthermodynamic assumptions. The simplest assumption, and the one we shall use here, is to set $a_{GuH^+} = a_{Cl^-} = a_\pm = a_{GuHCl}^{1/2}$.

It is well known that different salts of GuH^+ differ in their effectiveness as denaturants, as shown, for example, by Castellino and Barker (1968). Since this and similar studies are based on denaturant *concentrations*, part of the difference could be ascribable to differences in the activity of GuH^+ at a given concentration, *i.e.*, direct action by the anion is not essential to explain the observations. It is likely, however, that at least part of the effect is due to direct participation by the anion in the denaturing action of guanidinium salts. On the other hand, guanidinium salts are so much more effective as denaturing agents than salts of simpler cations (*e.g.*, NH_4^+) that the major part of the denaturing action must be ascribed to GuH^+ ion. Since GuHCl is a less effective denaturant than GuHBr, GuHI, and GuHSCN (Castellino and Barker, 1968), the anion term in eq 14 may in fact play a minimal role in determining $f(a_{GuHCl})$. In any event, to reduce the number of parameters in eq 14 we are compelled, as in eq 11, to use just a single binding constant, k'', for all interacting sites, giving

$$K_D/F(a_H) = K_D^0(1 + k''a_\pm)^{\Delta n} \quad (15)$$

and it is at least plausible that k'' and Δn refer to binding sites for GuH^+, those for Cl^- being among the weaker binding sites that make only a small contribution to the overall variation of K_D with GuHCl concentration.

[3] The activity of water at any concentration of GuHCl is the primary measurement in the isopiestic method. The activity of GuHCl as given by equation 2 is derived from the water activities. The complete measruements of E. P. K. Hade, Jr., will be published in the near future.

Application of the computer program to the data of Figure 1 gives the following values for the parameters of eq 15: $\Delta n = 21.5$, $k'' = 1.20$, and $\log K_D^0 = -6.65$. Within the activity range encompassed by the data of Figure 1, the curve representing eq 15 with these parameters is identical with the curve representing eq 11, as drawn in the figure. The two equations must of course deviate at lower GuHCl activities, since the values of K_D^0 differ substantially.

Equation 15 could be modified, as eq 11 was, to allow for competition between GuH^+ or Cl^- ions and water molecules. The result would again be a decrease in the best values of Δn and K_D^0.

By eq 3, 4, and 7, the action of GuHCl in promoting denaturation may be correlated with associated changes in the binding of the ions of GuHCl, or of water, or both. One way of bringing hydration into a model to quantitate this relation is to introduce competitive binding sites, as has already been done. Another way is to introduce sites specific for water alone. One possibility is that there may be sites on the surface of the native protein molecule, which accommodate water molecules but exclude ions in general.[4] Such sites would act as binding sites for water with very high affinity: for practical purposes they can be considered as combined with water, regardless of the concentration of GuHCl or other electrolytes. In view of the inherent flexibility of the randomly coiled form of a protein, sites that exclude ions are less likely to exist in the denatured protein, *i.e.*, the change in the number of such sites that accompanies denaturation is necessarily negative. If q such sites are lost, and, an additional Δn sites for GuH^+ or Cl^- are gained, eq 15 would become modified

$$K_D/F(a_H) = K_D^0(1 + k''a_\pm)^{\Delta n}/a_W^q \qquad (16)$$

Equation 16 can be fitted to the data of Figure 1 over a wide range of values for q. If, for example, we set $q = 30$, we obtain the following best values for the other parameters: $\Delta n = 9.85$, $k'' = 7.0$, $\log K_D^0 = -9.08$, *i.e.*, the effect of hydration sites of this kind is the expected one of reducing the number of binding sites for denaturant ions that are needed to account for the data of Figure 1. Formally, it would be possible to make q so large that Δn would be reduced to zero. This would be equivalent to ascribing the experimental values of $\Delta\bar{\nu}_{GuHCl,pref}$ *entirely* to negative values of $\Delta\bar{\nu}_W$. Such a solution to the problem would, however, be absurd, since it would make $K_D/F(a_H)$ a unique function of the activity of water alone, with the implication that any substance that reduces a_W would be an effective denaturing agent. This is contrary to experimental fact: many substances reduce a_W to the level it has in concentrated GuHCl solutions without causing any substantial conformational change in the native protein.

It should be noted in conclusion that the equations derived for the various binding mechanisms, with the values for the parameters assigned to them, are virtually indistinguishable within the range of GuHCl activity where experimental data are available. The standard deviation between experimental K_D values and K_D values computed by the equations is essentially the same (± 0.12 in log K_D) for each of the equations used. It is thus impossible, on the basis of these results, to consider any one of the proposed mechanisms as preferable, or to exclude numerous other possible equations for generating the experimental values of $\Delta\bar{\nu}_{GuHCl,pref}$.

Regardless of the equation employed, the results are consistent in indicating that Δn is relatively small compared with the number of amino acid residues. They also lead to fairly similar values of log K_D^0, in the range of -4 to -9. Higher credence should probably be given to the values of log K_D^0 of -6.6 to -9, obtained on the basis of ion binding, as the binding of GuHCl *molecules* is inherently an improbable event.

Discussion

The results of the analysis presented in this paper are somewhat disappointing in that they do not permit definite conclusions about the mechanism or site of action of GuHCl. On the basis of solubility studies of amino acids and peptides in GuHCl solutions (Y. Nozaki and C. Tanford, in preparation), it appears likely that the strongest binding sites for GuHCl or its ions on a protein molecule are (1) the aromatic side chains, and (2) pairs of adjacent peptide groups. The identification of the latter as binding sites for GuH^+ has also been proposed by Robinson and Jencks (1965). The results are not incompatible with the idea that these are indeed the principal binding sites responsible for GuHCl denaturation. The 12 aromatic side chains of lysozyme are unusually accessible to solvent in the native state (Williams *et al.*, 1965) so that they will constitute sites for the binding of GuH^+ in the native as well as in the denatured state and contribute perhaps no more than 2 or 3 sites to Δn. As for the peptide groups, they may not be as accessible in randomly coiled lysozyme (disulfide bonds intact) as in other denatured proteins, because the volume of the molecular domain is unusually small, the intrinsic viscosity of the denatured molecule being only 6 cc/g (Tanford, 1968). It is also possible that occupation of a pair of adjacent peptide groups by GuH^+ may interfere with binding of another GuH^+ to a second pair of peptide groups in the immediate neighborhood of the first bound ion. Thus a Δn value of about 20, ascribable to peptide binding sites for GuH^+, is not an impossible one. All these remarks are, however, speculative, and it must be concluded that the positive identification of the binding sites for GuH^+ (assuming that they exist) is still largely unsolved. More work with oligopeptides of varying length and with other proteins is necessary.

The K_D^0 values derived from the analysis *formally* represent the equilibrium constant for the reaction native protein $\rightleftharpoons$ cross-linked random coil, in the absence of denaturant, at pH $= -\infty$, and $K_D^0F(a_H)$ *formally* represents the same equilibrium constant, in the absence of denaturant, at other pH values. However, these extrapolated equilibrium con-

[4] The existence of sites of this kind is suggested by the experimental fact that native proteins in solutions of alkali halide salts are generally preferentially hydrated (Cox and Schumaker, 1961; Ifft and Vinograd, 1966; Hade and Tanford, 1967). Unpublished data of E. P. K. Hade, Jr., in 1 M GuHCl indicate that native proteins in this solvent preferentially bind GuHCl, though they do so, of course, to a much smaller extent than denatured proteins in GuHCl solutions. This may be the result of a combination of two factors: the existence of sites that accommodate water only *plus* sites that bind GuH^+, *e.g.*, exposed aromatic residues. The number of the latter in the native protein is not zero, though it is smaller than in the denatured protein.

TABLE I: Equilibrium Constants and Free Energies for Formation of the Randomly Coiled State in the Absence of Denaturant, at 25° and Neutral pH.[a]

Eq Used	Log K_D	ΔG_D^0 (kcal/mole)
11	−7.8	+10.6
13	−8.4	+11.5
15	−10.4	+14.2
16	−12.9	+17.6

[a] The values of log K_D^0 cited in the text refer to pH $= -\infty$, at high ionic strength. This is probably a hypothetical state, since a salt used to create a high ionic strength would generally itself affect ΔG_D^0.

stants correspond with the real K_D values under the stated conditions only insofar as the expression for $F(a_H)$ is applicable. The equations for $F(a_H)$ that we have used (paper I of this series) are actually valid only if long-range electrostatic forces exert a negligible influence on K_D, and therefore are not valid at low pH in the absence of denaturant unless a high concentration of a benign salt is present to maintain a high ionic strength. At higher pH, long range electrostatic forces should become unimportant, and $K_D^0F(a_H)$ values calculated from the data should quite closely represent actual K_D values in the absence of denaturant. Values so derived (for pH near neutrality), together with the corresponding free energies, are shown in Table I.

Table I shows, regardless of which of the given results proves to be correct, that the free energy of stabilization of lysozyme, in a native-like environment, is remarkably small. The same conclusion applies to other globular proteins. From studies of the denaturation of ribonuclease by GuHCl, for example, one concludes that ΔG^0 for formation of a random coil at 25° and pH 6 is about 10 kcal/mole (unpublished data, obtained in this laboratory by A. Salahuddin). The thermal denaturation of myoglobin, believed to lead to a less completely unfolded product than GuHCl denaturation (Tanford, 1968), is characterized by a ΔG^0 of 13.6 kcal/mole at 25° and pH 9 (Hermans and Acampora, 1967).

At acid pH, the stabilization of lysozyme is reduced even further. Equation 15, for example, gives log $K_D^0 = -6.6$, at 25° and pH < 1, which corresponds to $\Delta G^0 = 9$ kcal/mole. If the anticipated effect of long-range electrostatic repulsions (in the native state, at low ionic strength) is allowed for, the free energy of stabilization would become even less, of the order of 5–6 kcal/mole. Most other globular proteins also become less stable as the pH is reduced, and many undergo transitions to substantially disordered conformations by reduction of pH alone.

This inherently low stability of native globular structures is one of the most significant findings to emerge from denaturation studies in general. If we consider that a single buried aromatic side chain contributes about 3 kcal/mole to the stability of a native protein in water (Tanford, 1962), it becomes evident that a relatively small number of amino acid replacements can convert a native globular protein into a polypeptide chain that may not be able to adopt an ordered globular conformation at all. A possible implication is that polypeptide chains in general, with arbitrary amino acid compositions and sequences, will not possess stable ordered structures, and that the polypeptide chains that do form ordered proteins represent a special set of sequences, selected by evolutionary pressure. A somewhat more detailed statement of essentially the same conclusion has been given in a recent paper by Edsall (1968).

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