[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Thermodynamic Considerations of Protein Reactions.[1,2] II. Modified Reactivity of Primary Valence Bonds

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RECEIVED JUNE 11, 1956

The thermodynamic treatment of internal hydrogen bonding in proteins, previously applied to the modified reactivity of polar R groups and to other protein reactions, is extended here to include the modified reactivity of primary valence bonds, primarily peptide bonds but also disulfide bonds. It is shown that a primary valence bond in a protein may be apparently more stable than the corresponding bond in a low molecular weight model compound because of a contribution from the free energy required to break the hydrogen bonds between a given peptide fragment and the remainder of the protein molecule. This treatment may account for some of the observed differences in the behavior of proteolytic enzymes toward native and denatured protein substrates. Finally, a new method for studying the internal hydrogen bonding in proteins is suggested.

Introduction

In paper I[4,5] it was shown that the modified reactivity of side chain polar R groups of amino acid residues in proteins could be accounted for in terms of intramolecular hydrogen bonding between these groups. A thermodynamic treatment was presented to account for various anomalies observed in studies of the binding of protons and other low molecular weight ions and molecules by proteins. For example, the modified ionization behavior of the tyrosyl and carboxyl groups in serum albumin could be accounted for in terms of hydrogen bonding, an explanation which appears to be compatible with the *p*H dependence of the hydrodynamic behavior of this protein.[6,7] The hydrogen-bonded model was also applied to the polymerization of fibrin monomer, a process shown to involve the formation of hydrogen bonds between 19 histidyl acceptors and 19 donor groups which may be tyrosyls, lysyls or both.[8] As indicated in paper I, the effects of hydrogen bonding between the polar R groups should manifest themselves in all protein reactions where such hydrogen bonds are either formed or broken (*e.g.*, proteolysis, denaturation, enzyme reactions, etc.). In this paper the theory of paper I will be extended to the modified reactivity of primary valence bonds in proteins; it will be shown that such bonds may acquire an enhanced stability due to the hydrogen bonding.

The phenomenon of enhanced stability plays a role in reactions involving the liberation of a fragment of a protein molecule. Initially this fragment is considered to be attached to the remainder of the molecule by primary valence and hydrogen bonds which have to be broken in the reaction in order that the fragment be liberated. The free energy required to break the hydrogen bonds may render the primary valence bond *apparently stronger* than would be expected in the absence of such hydrogen bonds. For example, even though the hydrolysis of peptide bonds in low molecular weight model compounds is favored for the usual standard state of one mole per liter[9] (and even more so at the lower concentrations usually employed), it need not be so for *some* peptide bonds in a protein due to the additional stabilization provided by hydrogen bonds.

Two types of primary valence bonds in proteins, the peptide and disulfide bonds, can be broken readily, and occasionally quite specifically. This paper is limited to a consideration of such bonds. Because of mathematical complexity we can provide a quantitative treatment of only some aspects of those reactions in which *one* primary valence bond is broken; some additional qualitative statements will be made about the breakage of many bonds. The recent interest in many examples of limited proteolysis[10] suggests that several such systems may be available for a detailed thermodynamic study in the near future. It is also hoped that the thermodynamics of oxidation or reduction of individual disulfide bridges in proteins may soon be subjected to experimental investigation.

In the treatment of proteolysis presented herein we shall make use of the equilibrium constant for the hydrolysis of peptide bonds in model compounds, K_{pep}, and all of the constants characterizing the behavior of the polar R groups, these being the two ionization constants K_1 and K_2 of the non-hydrogen bonded donor and acceptor groups, respectively, and the hydrogen bonding constants K_{ij}, K_{1m} and K_{rs} (see paper I for the definitions of these equilibrium constants).

General Considerations

The protein model has been described in paper I. The molecule is considered as an assembly of helically folded peptide chains, some or all of which may be either open or cyclic. The chains are assumed to be held rigidly with respect to each other by a number of inter- and intra-chain disulfide bonds and by some inter- and intra-chain hydrogen bonds be-

(1) This work was done in connection with projects supported at Cornell University by the National Institutes of Health (U. S. Public Health Service), and by the Office of Naval Research. Reproduction in whole or in part is permitted for any purpose of the United States Government.

(2) Presented, in part, before the Division of Biological Chemistry at the 124th meeting of the American Chemical Society, Chicago, Illinois, September, 1953.

(3) U. S. Public Health Service Research Fellow of the National Heart Institute, 1952–1956.

(4) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(5) All equations herein designated I will be found in paper I.[4] This paper should also be consulted for the nomenclature of hydrogen bonding.

(6) H. A. Scheraga, G. I. Loeb and M. L. Wagner, *Federation Proc.*, **15**, No. 1138 (1956).

(7) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, in press.

(8) J. M. Sturtevant, M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, THIS JOURNAL, **77**, 6168 (1955).

(9) A. Dobry, J. S. Fruton and J. M. Sturtevant, *J. Biol. Chem.*, **195**, 148 (1952).

(10) N. M. Green and H. Neurath, "The Proteins," Ed. by Neurath and Bailey, Vol. IIB, Academic Press, New York, N. Y., 1954, p. 1183.

tween the polar R groups in the manner described in paper I.

We can distinguish between two types of primary valence bonds in a protein, those in closed and those in open loops[11] (see Fig. 1). The breakage of *one* "cyclic" bond can never cause a dissociation of the protein molecule into two fragments,[12] while breakage of one "non-cyclic" bond will, in general, yield two fragments.

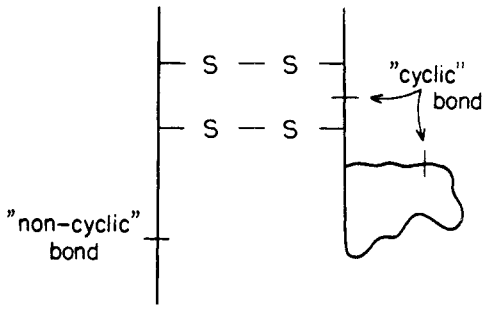

Fig. 1.—Examples of "cyclic" and "non-cyclic" bonds. The S–S bridges connect the polypeptide chains.

The thermodynamics of the breakage of "cyclic" bonds is very complex and will depend not only on the properties of the peptide bond and of the associated hydrogen bonds but also on the nature of the ring in which the "cyclic" bond participates, such as size, strain, etc. It is difficult to predict whether the breakage of such a bond will lead to a concomitant breakage of hydrogen bonds. The treatment here will, therefore, be limited to "non-cyclic" bonds with only a brief, qualitative reference to "cyclic" bonds. The most important thermodynamic difference between the cyclic and the non-cyclic cases is that the equilibrium extent of breakage for a "non-cyclic" bond generally depends on the original concentration of the protein, while it does not for the "cyclic" bond.

In the breakage of a primary valence bond in a non-cyclic chain it is necessary to decide whether this breakage is sufficient to cause the liberation of a given peptide fragment, since it is hypothetically possible that the hydrogen bonds would, *of themselves*, be sufficient to maintain the connection between the fragment and the "core." As an illustration, let us consider a hypothetical situation in which a fragment A is connected to the core C by a "non-cyclic" peptide bond and by a heterologous, single hydrogen bond[4] (see Fig. 2). For the sake of simplicity it will be assumed that the proteolytic reaction is studied at a *p*H where the acceptors exist predominantly in the form A and the donors in the form DH with no accompanying ionization. The situation is then representable by the four equilibria of Fig. 3 where (a) denotes the unhydrolyzed, hydrogen bonded protein, (b) the unhydrolyzed, non-hydrogen bonded protein, (c) the hydrolyzed, hydrogen bonded protein and (d) the hydrolyzed, non-hydrogen bonded protein, *i.e.*, the free fragment A and the core C. The equilibrium

(11) For convenience we shall refer to bonds in closed loops as "cyclic" bonds and those in open loops as "non-cyclic" bonds.

(12) We are excluding from consideration protein molecules which are composed of fragments held solely by secondary bonds. In such a molecule, breakage of any bond in a fragment may lead to dissociation.

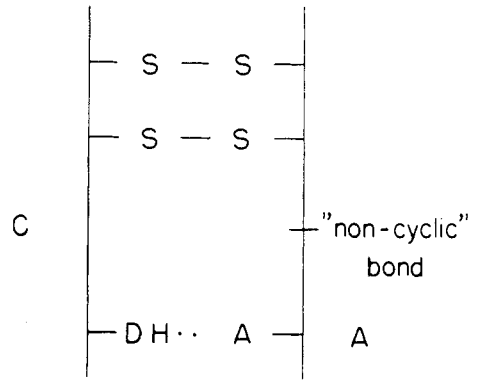

Fig. 2.—Connection of fragment A to core C by a "non-cyclic" bond and a heterologous, single hydrogen bond.

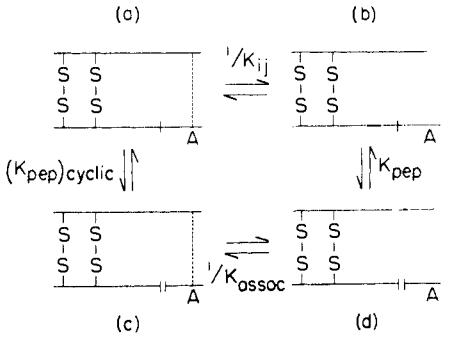

Fig. 3.—Equilibria among various species. The single, solid vertical line represents a non-hydrolyzed peptide bond and the double, solid vertical lines represent a hydrolyzed peptide bond. The fragment to be liberated is designated A. The dashed, vertical line represents a hydrogen bond.

constant K_{assoc} for the association of A and C differs from K_{ij}, since the latter is the constant for the formation of an internal hydrogen bond between the i^{th} donor and j^{th} acceptor in a single molecule, whereas the former applies to a bimolecular association reaction. Of the four equilibrium constants used in Fig. 3, only three are independent and must be known in order to completely define the state of the system.

Approximate values of two of these constants are available; K_{pep} is the hydrolysis constant for a peptide bond in an appropriate model compound, and K_{ij}, the internal hydrogen bonding constant, has been defined, discussed and evaluated approximately in paper I. Much less information is available about the size of $(K_{pep})_{cyclic}$ and K_{assoc}. These equilibrium constants are probably so strongly dependent upon the number, nature and geometric arrangement of the hydrogen bonds holding the fragment A to the core C that it is unprofitable even to speculate about the range of orders of magnitude of these constants. In at least one case, however, Herriott[13] has shown that K_{assoc} is appreciable. The autocatalytic activation of pepsinogen proceeds according to the following mechanism.[13,14]

$$\text{Pepsinogen} \underset{(?)}{\rightleftarrows} \text{Pepsin–inhibitor compound} \rightleftarrows \text{Pepsin} + \text{Inhibitor}$$

(13) R. M. Herriott, *J. Gen. Physiol.*, **22**, 65 (1938); **24**, 325 (1941).

(14) The symbol $\underset{(?)}{\rightleftarrows}$ in the first step has been inserted by the present authors.

In the first step peptide bonds are broken, while the second involves the rupture of secondary bonds only. At *p*H values higher than 5.4 the equilibrium in the second step, under Herriott's experimental conditions, is shifted to the left, while below *p*H 5.4 it is shifted to the right, thus showing that K_{assoc} is quite appreciable and strongly *p*H dependent.

The existence of an appreciable K_{assoc} causes considerable difficulty in the study of the equilibria of Fig. 3. It becomes important to decide what is really being measured in an experimental investigation of the course of proteolysis. The two alternatives are (1) the amount of peptide fragment A and of core C liberated or, (2) the amount of peptide bonds broken. These two quantities differ by the amount of the hydrogen bonded core-fragment complex (c). If experimental methods to follow alternative 1 were in common use there would be no further difficulty in defining and obtaining the observed hydrolysis constant $(K_{pep})_{obs}$ from the equilibrium constants of Fig. 3. In such a case $(K_{pep})_{obs}$ would be defined by

$$(K_{pep})_{obs} = \frac{(d)^2}{(a) + (b) + (c)} \qquad \text{(II-1)}$$

and since[15]

$$K_{ij} = \frac{(a)}{(b)} \qquad \text{(II-2)}$$

$$K_{assoc} = \frac{(c)}{(d)^2} \qquad \text{(II-3)}$$

$$K_{pep} = \frac{(d)^2}{(b)} \qquad \text{(II-4)}$$

it readily follows that

$$1/(K_{pep})_{obs} = K_{assoc} + (1 + K_{ij})/K_{pep} \qquad \text{(II-5)}$$

However, almost all of the present methods of studying proteolysis do not follow the liberation of peptide fragments, but instead indicate the breakage of peptide bonds. This is apparent in various titrimetric techniques, observing the appearance of new titratable groups, but it is also probably true of such techniques as non-protein nitrogen or chromatographic analysis of resulting peptides. In the latter methods the separation of peptide products is accomplished by such drastic means (*e.g.*, trichloroacetic acid precipitation) that K_{assoc} must be strongly affected and almost all of the peptide held must be released. Herriott's method for estimation of pepsin activity in the pepsinogen–pepsin system follows our alternative 1, but even then the amount of free pepsin was measured only because the rate of dissociation of the pepsin–inhibitor compound is very small and thus does not affect the pepsin assay.

Unfortunately, no convenient definitions of $(K_{pep})_{obs}$ on the basis of alternative 2 are possible. The only alternative available, in the absence of further information, is to *assume* that in many situations K_{assoc} will be negligibly small and hence species (c) does not exist, *i.e.*, that the hydrogen bonds alone will not be able to hold a peptide fragment to the protein in a proteolytic reaction. Such an assumption appears at first quite reasonable; however, the possibility of protein association by means of hydrogen bonds and the case of the pepsin–inhibitor compound render it less plausible. The validity of this assumption should always be tested in a given experiment by attempting to measure $(K_{pep})_{obs}$ over a wide range of concentrations. If this assumption holds, $(K_{pep})_{obs}$ will be independent of concentration.

The treatment to be presented herein will be based on the assumption that K_{assoc} is negligibly small. Hence, under this assumption, the proteolytic reaction will be envisioned as involving not only the breakage of the peptide bond but also the concomitant breakage of the hydrogen bonds between the fragment A and the core C. It will be shown that it is the free energy required to break the hydrogen bonds which may provide the stabilization of the peptide bond against hydrolysis.

Thermodynamic Formulation

If K_{assoc} is assumed to be zero, the equilibria of Fig. 3 are greatly simplified, and reduce to those of Fig. 4. In such a case the observed equilibrium

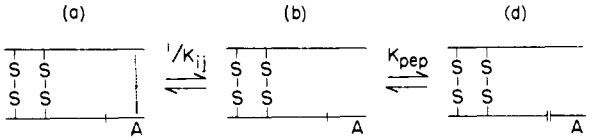

Fig. 4.—Simplification of the equilibria of Fig. 3, assuming no core-fragment complex.

constant for the hydrolysis of the peptide bond, $(K_{pep})_{obs}$, is given by the following simplified form of eq. II-5

$$(K_{pep})_{obs} = \frac{K_{pep}}{1 + K_{ij}} \qquad \text{(II-6)}$$

In general, however, the fragment A will be held to the core by more than one hydrogen bond. Further, the donors DH and acceptors A may ionize and, thereby, affect the hydrogen bonding equilibria. In order to obtain a more general expression we shall define quantities x_{ij}, which are the concentration fractions of all non-hydrolyzed protein molecules containing a hydrogen bond between the i^{th} donor and the j^{th} acceptor. This quantity is equal to $P_{DH \ldots A}$ of paper I. By the method of Appendix I of paper I it follows that, for a heterologous, single hydrogen bond

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \qquad \text{(II-7)}$$

if we neglect the small terms K_1/K_2 and $K_{ji}K_1/K_2$. In this expression K_{ij} is the usual hydrogen bonding constant and K_1 and K_2 are the ionization constants of the non-hydrogen bonded donor and the acceptor, respectively.

We shall regard K_{pep} not only as the hydrolysis constant for a peptide bond in a simple, non-hydrogen bonded model compound but also as the value to be expected for the particular bond in a protein in which no hydrogen bonds exist between the fragment A and the core C. In other words, we regard the peptide bond in a protein as a normal bond with no intrinsically different properties aris-

(15) For simplicity, and in conformance with current usage, the concentration of H_2O has been omitted from the expressions for the hydrolysis equilibrium constants.

ing from the fact that it is in a protein instead of a model compound. We then have

$$K_{pep} = \frac{(C)(A)}{(P_{NHB})} \qquad (II\text{-}8)$$

where (P_{NHB}) is the concentration of the non-hydrolyzed protein assuming that it contains no hydrogen bonds between the R groups, and (C) and (A) are the concentrations of the protein core and peptide fragment, respectively.

The *observed* hydrolysis constant for the given peptide bond in the protein, in the presence of the stabilizing hydrogen bonds, is then defined as

$$(K_{pep})_{obs} = \frac{(C)(A)}{(P)} \qquad (II\text{-}9)$$

where (P) is the *total* concentration of non-hydrolyzed protein, both with and without hydrogen bonds. If we let $(P_{HB})_{ij}$ be the concentration of non-hydrolyzed protein which contains a hydrogen bond between the i^{th} donor and j^{th} acceptor, then, by definition

$$(P_{HB})_{ij} = x_{ij}(P) \qquad (II\text{-}10)$$

and

$$(P_{NHB})_{ij} = (1 - x_{ij})(P) \qquad (II\text{-}11)$$

Let us assume that the hydrogen bonds are non-competitive and non-coöperative (see paper I for a description of these types of hydrogen bonds), and, therefore, independent of each other. Then

$$(P_{NHB}) = (P)\pi(1 - x_{ij}) \qquad (II\text{-}12)$$

where the product is taken over all the possible hydrogen bonds between the core and the peptide fragment. Combining eqs. II-8, II-9 and II-12 we obtain

$$(K_{pep})_{obs} = K_{pep}\pi(1 - x_{ij}) \qquad (II\text{-}13)$$

This result shows the influence of hydrogen bonding between the segment A and the core C upon the hydrolysis constant $(K_{pep})_{obs}$, *i.e.*, the presence of the hydrogen bonds influences the hydrolysis of the peptide bond because of the "pull" of the consecutive equilibria outlined in Fig. 4. For various hydrogen bonding situations different values of x_{ij} can be derived readily by the method of Appendix I of paper I and inserted into eq. II-13; *e.g.*, for heterologous, single hydrogen bonds x_{ij} is given by eq. II-7. Equation II-13 thus shows how the stability of the peptide bond is modified by the hydrogen bonds, since $(K_{pep})_{obs}$ is less than K_{pep} because $(1 - x_{ij})$ is less than unity.

Some question may arise here as to the correctness of the use of K_{pep}, obtained from small model compounds, for describing the properties of "normal" peptide bonds in proteins. Aside from the possible, although probably negligible, difference between the peptide bonds in long peptide chains and in small compounds, two main differences are also neglected. First, if the protein chain is helical, several peptide NH–OC hydrogen bonds must be broken along with the peptide bond. No information on the strength of such peptide hydrogen bonds and thus on the magnitude of this effect is available.[16] Further, even though the model compounds cannot be helically folded, they too (except the simplest) could conceivably assume configurations giving rise to intramolecular hydrogen bonds[17] between the peptide NH and CO groups. Such bonds would be broken by hydrolysis as well. The second effect arises from the great number of charges generally carried by a protein. The separation of these charges occurring during hydrolysis will give rise to an electrostatic free energy term, which could either enhance or reduce hydrolysis.[18]

The only item of experimental evidence with a bearing on these problems is the finding of Sturtevant[19] that the average heat of hydrolysis of peptide bonds in polylysine is roughly the same as the heats of hydrolysis of peptides.

From equation II-13 for $(K_{pep})_{obs}$ we can deduce the corresponding thermodynamic parameters

$$(\Delta F^\circ_{pep})_{obs} = -RT \ln (K_{pep})_{obs} = \Delta F^\circ_{pep} - RT \Sigma \ln (1 - x_{ij}) \qquad (II\text{-}14)$$

$$(\Delta S^\circ_{pep})_{obs} = \frac{-\partial(\Delta F^\circ_{pep})_{obs}}{\partial T} = \Delta S^\circ_{pep} + R \Sigma \ln (1 - x_{ij}) + RT \Sigma \frac{\partial \ln (1 - x_{ij})}{\partial T} \qquad (II\text{-}15)$$

$$(\Delta H^\circ_{pep})_{obs} = (\Delta F^\circ_{pep})_{obs} + T(\Delta S^\circ_{pep})_{obs} = \Delta H^\circ_{pep} + RT^2 \Sigma \frac{\partial \ln (1 - x_{ij})}{\partial T} \qquad (II\text{-}16)$$

These expressions are not simple, since they include the distribution among a large number of species. It is probably more instructive to consider the approximate expressions for the case of heterologous, single hydrogen bonding between non-ionizable donors and acceptors. This restriction simplifies eq. II-7 to

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij}} \qquad (II\text{-}17)$$

(16) Some rough estimates of the free energy of formation of NH...OC peptide hydrogen bonds were made by J. A. Schellman, *Compt. rend. trav. lab., Carlsberg*, **29**, No. 15, 230 (1955). Schellman estimated the entropy and enthalpy of formation of such a hydrogen bond as considerably less negative than our values[4] for the hydrogen bond between the polar R groups. Thus, due to a compensation, the free energies of formation are quite similar. However, the Schellman estimate appears to be inconsistent, since the effect of water appears to be included in the enthalpy term and neglected in the entropy (this effect is neglected throughout in our treatment). In addition, it should be noted that in water the strength of the NH...OC bonds need not be directly comparable to the strength of polar R group hydrogen bonds due to (1) the resonance contribution of the N=C—OH form to the peptide bond, (2) the possibility that a different amount of energy may be required to rotate the single bonds of the peptide chains into a helical configuration[17] compared to the energy required to rotate the single bonds in the R groups into the hydrogen bonded configuration, and (3) the difference in the moments of inertia associated with libration around single bonds in the peptide chain as compared to the R groups. It is, however, worth noting that Schellman's value for the enthalpy of formation of a peptide hydrogen bond is based on the heat of dilution of aqueous urea whereas our estimate for a polar R group hydrogen bond has been confirmed, as will be shown below, by some experiments on two *proteins*, serum albumin and monomeric fibrin. Despite these differences in estimated values for the two types of hydrogen bonds, Schellman's general conclusion that a polypeptide chain must have a critical size to be in a helical configuration is valid. What is still undetermined is the numerical value of the critical size. For simplicity, we are assuming in our treatment of proteolysis here that the fragment A is large enough to maintain its helical configuration when it is liberated from the core C. Hence, for an α-helix, the only NH...OC peptide hydrogen bonds broken in the hydrolysis of one peptide bond are the 4 near the ends of the polypeptide chain.

(17) S. Mizushima, "Structure of Molecules and Internal Rotation," Academic Press, New York, N. Y., 1954.

(18) An additional charge effect, referred to in footnote 24 of paper I, can arise if the breakage of hydrogen bonds accompanying proteolysis leads to a change in the state of ionization of a particular R group. Such charge effects would give rise to volume changes accompanying proteolysis which could also be treated with our model in the same manner as the other thermodynamic quantities to be discussed below. Some discussion of the volume changes accompanying ionization was presented in paper I.

(19) J. M. Sturtevant, THIS JOURNAL **77**, 1495 (1955).

so that eqs. II-14, II-15 and II-16 become

$$(\Delta F^\circ_{pep})_{obs} = \Delta F^\circ_{pep} + RT \Sigma \ln (1 + K_{ij}) \quad \text{(II-18)}$$

$$\begin{aligned}(\Delta S^\circ_{pep})_{obs} &= \Delta S^\circ_{pep} - R \Sigma \ln (1 + K_{ij}) \\ &\quad - RT \Sigma \frac{\partial \ln (1 + K_{ij})}{\partial T} \\ &= \Delta S^\circ_{pep} - R \Sigma \ln (1 + K_{ij}) \\ &\quad - RT \Sigma \frac{K_{ij}}{1 + K_{ij}} \frac{\partial \ln K_{ij}}{\partial T} \\ &= \Delta S^\circ_{pep} - R \Sigma \ln (1 + K_{ij}) \\ &\quad - \frac{1}{T} \Sigma \frac{K_{ij}}{1 + K_{ij}} \Delta H^\circ_{ij} \quad \text{(II-19)}\end{aligned}$$

$$(\Delta H^\circ_{pep})_{obs} = \Delta H^\circ_{pep} - \Sigma \frac{K_{ij}}{1 + K_{ij}} \Delta H^\circ_{ij} \quad \text{(II-20)}$$

These expressions are analogous to eqs. I-38, I-39 and I-41 for the modified ionization behavior of donor groups involved in heterologous, single hydrogen bonds.

The positive term $RT \Sigma \ln (1 + K_{ij})$ in eq. II-18 is the free energy of stabilization of the peptide bond in the protein, arising from the hydrogen bonding. In the next section we shall discuss the magnitude which this stabilization may attain.

Magnitude of Peptide Bond Stabilization

It is now of interest to compute the magnitude of the stabilization in order to see whether the effect of the hydrogen bonding can be observed experimentally. Since K_{pep} is quite large in model compounds,[9] the hydrolysis of peptides (at least at low molarities) proceeds essentially to completion. Since the deviation of the equilibrium position from the stoichiometrically complete one frequently cannot be observed it may be impossible to measure a small change in $(K_{pep})_{obs}$. To find out the conditions under which such an equilibrium may be observed experimentally in protein hydrolysis, let us consider a protein with a molecular weight M and solubility S (in g./100 ml.). The molarity of a solution of such a protein is $10S/M$. If the protein can undergo a limited proteolysis leading to the formation of a single liberated fragment, the equilibrium constant in such a reaction is given by equation (II-9), which can be rewritten as

$$(K_{pep})_{obs} = \frac{\alpha^2 (P)_0}{1 - \alpha} \quad \text{(II-21)}$$

where $\alpha = (C)/(P)_0 = (A)/(P)_0$, and $(P)_0$ is the initial molar concentration of the protein. Let us further assume that experimental methods are available to distinguish α from 1, if it is equal to or smaller than 0.9. In such a case we can write

$$(K_{pep})_{obs} = \frac{\alpha^2}{1 - \alpha} \frac{10S}{M} \leqq \frac{81S}{M} \quad \text{(II-22)}$$

in order for $(K_{pep})_{obs}$ to be measurable. Since the order of magnitude of M for commonly studied proteins is 10^4 to 10^6 and of S is 1 to 10, $(K_{pep})_{obs}$ must be smaller than 10^{-1} to 10^{-4} in order to be measurable. Since[9] K_{pep} is probably between 1 and 10 it follows that $\pi(1 - x_{ij})$ must be smaller than 10^{-1} to 10^{-5} for the reversibility to be detectable. For a heterologous, single hydrogen bond $K_{ij} = 1$ (see paper I), hence $x_{ij} = 0.5$ (see eq. II-17), and thus $(1 - x_{ij})$ is also 0.5. Thus, for $\pi(1 - x_{ij})$ to be smaller than 10^{-1} to 10^{-5}, *at least* 4 to 17 heterologous single, hydrogen bonds must unite C and A. Therefore, one should expect that unless heterologous, single hydrogen bonds with a very high K_{ij} are involved, "apparently strong" peptide bonds would be observed only if the peptide fragment split off were quite large and contained a large number of polar residues, thus furnishing many sites for hydrogen bonding. Alternatively, fewer (1 or 2) homologous, double hydrogen bonds (*e.g.*, COOH–COOH acetic acid dimer type bonds), with $K_{1m} \sim 100$ would suffice to give a sufficiently stabilized peptide bond so that the reversibility would be detected experimentally. Preliminary results indicate that such may be the case in the proteolytic action of thrombin on fibrinogen.[20] However, it should be noted that whenever the number of hydrogen bonds uniting the fragment A and core C is large the chance that K_{assoc} is not negligible probably also increases greatly. In such a case the approximations of this section break down and it is no longer necessary that hydrolysis of a peptide bond should lead to a breakdown of the hydrogen bonds between the fragment and the core. If K_{assoc} is not negligible then cyclic structures will be involved in the stabilization. As already pointed out, the constancy of $(K_{pep})_{obs}$ over a range of concentration should be checked in such experiments.

It should also be noticed that, since $\Delta H^\circ_{ij} \sim -6$ kcal./mole,[21] $(\Delta H^\circ_{pep})_{obs}$ from eq. II-20 *will* show a measurable effect. Hence, the heat of hydrolysis of a peptide bond in a protein should provide the most stringent criterion of modified reactivity. A direct calorimetric measurement of the heat of hydrolysis may indicate a hydrogen bonding contribution even in cases where the number of hydrogen bonds is much too small for the reversibility to be detected in terms of an abnormal equilibrium constant.

Role of Denaturation

Since the enhanced stabilization of the peptide bond is envisaged as arising from the hydrogen bonds, and since the breakage of such hydrogen bonds may be regarded as a denaturation, it is seen that there is, in effect, a denaturation accompanying the hydrolysis of a stabilized peptide bond. This denaturation is involved here as a thermodynamic consequence of the model for proteolysis, and need not be considered as arising from some special "denaturase" activity of the proteolytic enzyme as suggested by Linderstrøm-Lang.[23,24] Further, it follows from our model that if a protein containing a stabilized peptide bond is denatured before being

(20) T. H. Donnelly, M. Laskowski, Jr., and H. A. Scheraga, A. C. S. abstracts, Sept. 1955, p. 23C.

(21) This value of ΔH^0_{ij} has now been confirmed experimentally both for the tyrosyl hydrogen bonds in serum albumin[4,22] and for the histidyl hydrogen bonds involved in the polymerization of fibrin monomers.[8]

(22) C. Tanford and G. L. Roberts, THIS JOURNAL, **74**, 2509 (1952).

(23) K. Linderstrøm-Lang, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 117 (1949).

(24) Some evidence against this assumption was, however, recently provided by C. H. Li, H. Papkoff, P. Fønss-Bech and P. G. Condliffe, *J. Biol. Chem.*, **218**, 41 (1956). These authors suggest that chymotrypsin may have a "denaturase" activity toward hypophyseal growth hormone independent of its proteolytic activity. No comment can be made until the nature of this important activity is further elucidated.

subjected to the action of the proteolytic enzyme, then the same peptide bond in the denatured protein should no longer be stabilized and hydrolysis should proceed with a large equilibrium constant as in model compounds. In addition to this thermodynamic consequence, we may also make a *guess* as to the kinetics by stating that denatured proteins should be hydrolyzed faster than their corresponding native forms. Finally, as seen from eq. II-13, the difference between $(K_{pep})_{obs}$ and K_{pep} disappears as the number of hydrogen bonds decreases. Thus it may happen that the first peptide bond hydrolyzed in a protein may be very strong but the subsequent peptide bonds being hydrolyzed may be weaker because the hydrogen bond stabilization is removed during the hydrolysis of the first peptide bond. One should then observe almost a simultaneous hydrolysis of the several peptide bonds in a more generalized proteolysis than the limited proteolysis heretofore considered. This phenomenon has been observed and referred to as a proteolytic explosion.[25]

Cyclic Stabilization

There is also an alternative explanation for the existence of apparently strong peptide bonds and proteolytic explosion in proteins which consist of a single, closed polypeptide chain, wherein hydrogen bonding need not play a dominant role. The first peptide bond, or even possibly the first several peptide bonds to be hydrolyzed[26] may yield no free fragments and thus no concomitant entropy gain from the liberation of free fragments. Thus, the first few bonds may appear stronger (as mentioned before, the entropy change in the breakage of cyclic bonds is likely to depend upon the properties of the individual ring and cannot be evaluated for a general case). The subsequent peptide bonds of the formerly cyclic chain may then be weaker (assuming hydrogen bonding to be absent) since the peptide is no longer cyclic and free fragments may then be liberated on hydrolysis. We can distinguish between the two extreme types of apparently strong bonds discussed here by a study of the dependence of the equilibrium extent of hydrolysis α on concentration (in those cases where the peptide bond is stabilized enough so that α is measurably less than 1), by end group analysis to indicate the presence of cyclic chains, or by the value of $(\Delta H^\circ_{pep})_{obs}$. For strong bonds in a cyclic peptide (in which we shall assume no hydrogen bonding) $(\Delta H^\circ_{pep})_{obs}$ will be the same as that for simple peptides, ΔH°_{pep}, which is slightly negative.[9] However, for the noncyclic case previously discussed, where the stability arises from internal hydrogen bonds, the value of $(\Delta H^\circ_{pep})_{obs}$ will be augmented by a contribution from the breakage of these hydrogen bonds (given in eq. II-20), and will be quite positive.

Concluding Remarks

It should be mentioned that the strength of other primary valence bonds, such as S–S bonds, in proteins should also be affected in a similar manner by hydrogen bonds. Thus, one should expect an apparent increase in the stability of these bonds if their rupture would also involve hydrogen bond breakage. The oxidation–reduction potentials of disulfide bonds in proteins should, therefore, be affected.

The observation that primary valence bonds may owe some additional thermodynamic strength to the hydrogen bonds associated with them, provides a new method for studying the internal structure of proteins. Measurements of hydrolysis constants (and associated enthalpy and volume changes) for peptide bonds in native proteins, as well as measurements of oxidation–reduction potentials of disulfide links should yield direct thermodynamic information about the number, nature and specific location of intramolecular hydrogen bonds between polar groups. This method should have an advantage over the presently used study of ionization constants, since it will yield data on hydrogen bonds involving non-ionizable groups (*e.g.*, serine and threonine) and about hydrogen bonds which cannot influence the ionization constants (*e.g.*, homologous single bonds[4]). Further, this method would be affected by the ambiguities in the interpretation of titration curves only as a second-order correction. An investigation of the structure of insulin from the point of view of locating the internal hydrogen bonds is currently in progress.[27]

It is worthwhile to restate here the suggestion of Levy and Slobodiansky[28] that the formation of hydrogen bonds in the end stages of protein synthesis influences the formation of the final peptide bonds. Since, at these final stages, the reacting compounds must necessarily be at very low molar concentrations (due to their high molecular weight) the synthesis would be very unlikely unless a favorable free energy situation could arise because of the presence of some other process, such as the formation of hydrogen bonds.

In the realm of pure speculation it is possible that some part of the great structural specificity of proteins is due to the fact that only molecules with considerable intramolecular hydrogen bonding can be synthesized.

It is of interest to point out that in limited proteolysis[10] (*e.g.*, the activation of zymogens), the release of a polypeptide may uncover hydrogen bond donor or acceptor sites which could play a role in the subsequent biological function of the resulting molecule. For example, the release of fibrinopeptide caused by thrombin[29,30] is believed[8,20,31] to uncover donor sites which are then available for the subsequent polymerization of fibrin monomer by means of a hydrogen bonding mechanism.

(25) A. Tiselius and I. B. Eriksson-Quensel, *Biochem. J.*, **33**, 1752 (1939).

(26) Such a situation could exist if the cyclic chain is heavily cross linked with disulfide bridges.

(27) M. Laskowski, Jr., J. M. Widom, M. L. McFadden and H. A. Scheraga, *Biochim. Biophys. Acta*, **19**, 581 (1956).

(28) M. Levy and E. Slobodiansky, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 113 (1950).

(29) L. Lorand, *Physiol. Revs.*, **34**, 742 (1954).

(30) K. Bailey and F. R. Bettelheim, *Biochim. Biophys. Acta*, **18**, 495 (1955).

(31) T. H. Donnelly, M. Laskowski, Jr., N. Notley and H. A. Scheraga, *Arch. Biochem. Biophys.*, **56**, 369 (1955).

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