Determination of the Number of Hydrogen-Bonds in a Protein Molecule

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By means of an infrared-spectroscopic observation of the hydrogen-exchange reaction, the number of peptide-peptide hydrogen-bonds involved in a given protein molecule in its aqueous solution can be determined.

Every globular protein is considered to involve a proper set of intra-molecular hydrogen-bonds in its native form, both in its crystalline state and in its aqueous solution. Such a proper set of the hydrogen-bonds seems to keep a proper rigidity and at the same time a proper flexibility of the protein molecule, both of which are essential for playing its biological role.

We propose here a simple and reliable method of determining the number of the peptide-peptide hydrogen bonds in a given protein molecule in its aqueous solution. The method is based on a hypothesis, which is supported by our recent experiments,[1-3] that the slowly exchanging peptide hydrogen atoms are assignable to those involved in such hydrogen bonds. Similar but slightly different hypotheses on the slowly exchanging hydrogen atoms in proteins seem to have for long been kept in minds by a number of investigators.[4-7] Until recently, however, they were not used for obtaining useful pieces of information of the protein structure. A tritium-gel filtration study recently made by Englander and Staley[8] demonstrated, for example, that the amount of slowly exchanging hydrogens found in myoglobin matched the known number of hydrogen-bonded amides.[9] While, in the measurement of ^3H radioactivity, one needs to take, in principle, all the exchangeable hydrogen atoms in the protein molecule in question into account, we can extract only the behavior of the peptide NH groups by an absorption measurement.[5] This is what we propose here to adopt.

The proposed method is as follows. Dissolve, at time zero, a protein into D_2O in 2~3% in concentration, and place the solution into a sealed cell with CaF_2 windows and of 50 μ in optical path length. Measure the time-dependent decrease in the ratio of the peak intensity of amide II band around 1540 cm^{-1} to that of amide I band at about 1650 cm^{-1} (A(amide II)/A(amide I)) (see Fig. 1). In the intensity measurements, the baseline for the amide I band is drawn parallel to the 100% transmittance line from the transmittance at 1800 cm^{-1}, and the baseline of the amide II band is taken as the absorption of a solution of the completely deuterated protein. Complete deuteration is achieved by heating the solution of the exchanging protein to a proper temperature, or by elevating the pH of the solution to 10 or so. Figure 2 illustrates a set of semilogarithmic plots of such a hydrogen→deuterium exchange reaction. The protein here is bovine pancreatic α-chymotrypsin. As may be seen in the Figure, two kinds of peptide hydrogen atoms are readily found: those which exchange quickly and those which do slowly. At 25 °C and at a proper pH, the exchange reaction of the former usually completes within three hours or so and that of the latter usually takes place

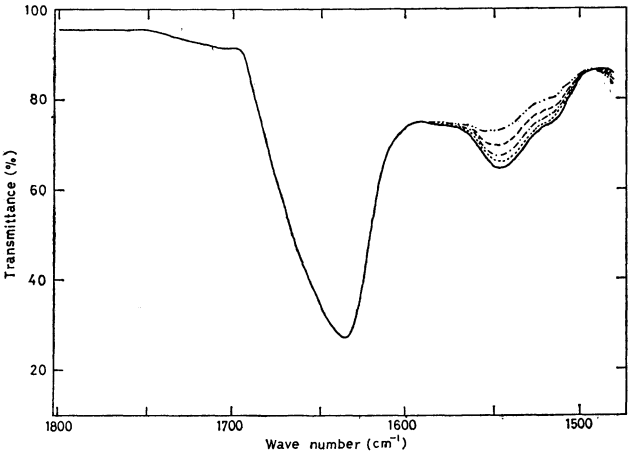

Fig. 1. Infrared absorption curves of bovine pancreatic α-chymotrypsin in D_2O solution of pH 3.5 at 35°C. —— 10 min, ····· 19 min, —·—·— 55 min, ---- 170 min, —··—··— 20 hr.

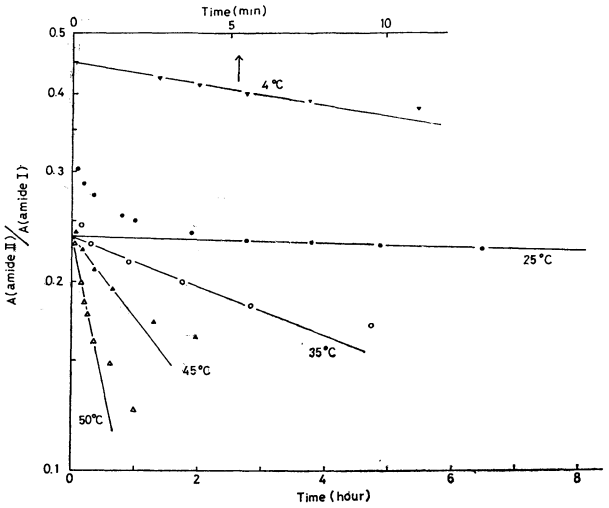

Fig. 2. Semilogarithmic plots of hydrogen → deuterium exchange reaction of bovine pancreatic α-chymotrypsin in D_2O solutions of pH 3.5 at various temperatures.

for one day or so. The semilogarithmic plot corresponding to the latter forms usually a straight line at least for first several hours or at least for first several points of measurement. Extend, then, this straight line till the ordinate axis and find the ordinate value for slowly exchanging peptide NH groups at time zero. Repeat similar kinetic measurements at different temperatures (from 10 °C to denaturation temperature, for example), at different pH's (from 4 to 8, for example), and/or with different concentrations of a denaturation agent (LiCl, for example, from 0

TABLE 1. DETERMINATION OF THE NUMBER OF PEPTIDE-PEPTIDE HYDROGEN-BONDS IN PROTEIN MOLECULES

Protein	Intensity ratio Amide II/Amide I at time zero		Ratio of NH···OC / total peptide NH's	Total peptide NH's	Peptide NH's involved in peptide-peptide hydrogen bonds	
	Total peptides (a_t)	Slowly exchanging peptides (a_i)	(a_i/a_t)	(N_t)	Hydrogen exchange experiment (N_i)	Found in X-ray analysis
Lysozyme	0.450	0.155[1,2]b)	34.4%	126[10]b)	44[1,2]	44[18]
Myoglobin	0.450	0.335[3]	74.4	148[11]	109[3]	108[9]
α-Chymotrypsin	0.450	0.235	52.2	229[12]	120	121[19]
Bovine pancreatic trypsin inhibitor	(0.450)a)	0.170	37.8	53[13]	20	20[20]
Concanavalin A	(0.450)a)	0.175	38.9	226[14]	88	(86)c)
α-Lactalbumin	0.450	0.130	28.9	120[15]	35	
Soy-bean trypsin inhibitor (Kunitz)	(0.450)a)	0.220	48.9	170[16]	83	
Streptomyces subtilisin inhibitor (17)	(0.450)a)	0.140	31.1	104[17]	33	

a) The a_t value was not determined for this protein. The value was assumed to be 0.450. b) The numbers in these parentheses indicate references. c) Counted on the basis of the description of Edelman *et al.* in the Ref. 14.

to 8 M). It is essential to confirm that the straight lines corresponding to the slowly exchanging hydrogen atoms converge into the same point on the ordinate axis (see Fig. 2). Record the ordinate value of this point as $[\text{A(amide II)/A(amide I)}]_i=a_i$ (given in the third column of Table 1). Next, the A(amide II)/A(amide I) value for the total peptide NH groups of this protein is to be determined. This is done by following very slow hydrogen→deuterium exchange reaction of the protein at pH=3 and/or at a low temperature (0~5 °C). Obtain the ordinate value at time zero by an extrapolation (see Fig. 2), and let it be $[\text{A(amide II)/A(amide I)}]_t=a_t$. For every protein so far examined, the a_t value has been found to be 0.450 (the second column of Table 1). From the result of amino acid analysis of the protein in question, find the total number N_t of peptide NH groups in the molecule. Then, $N_i=N_t(a_i/a_t)$ is considered to be equal to the number of peptide NH groups involved in peptide-peptide (NH···O=C) hydrogen bonds.

The method has been tested for five proteins, for which X-ray crystallographic analyses have been made. The results are shown in Table 1. As may be seen here the N_i values obtained by the proposed method are in accord with the numbers of peptide-peptide hydrogen bonds found by the X-ray analyses within ±1. The method has also been applied to bovine α-lactalbumine, soy-bean trypsin inhibitor (Kunitz), and Streptomyces subtilisin inhibitor. The N_i value found here (respectively 35, 83, and 33) are considered to be predicted numbers of peptide-peptide hydrogen bonds in these proteins.

The method involves an assumption that the contributions of various undeuterated peptide groups in the protein molecule to the A(amide II)/A(amide I) value are equal. This assumption is justified to some extent by our finding[21] that the intensity ratio now in question is practically independent of the helix content for poly-L-glutamic acid in an aqueous system. The assumption is all indirectly supported by our test for five proteins already mentioned.

It is well known that the position of the amide II band is slightly different for different polypeptide conformations (at 1535 cm^{-1} for the random conformation, at 1546 cm^{-1} for the α-helix, and at 1530 cm^{-1} for the β-structure). The amide I band also appears at different frequencies according to the conformations[22] (between 1650 and 1655 cm^{-1} for random, at 1650 cm^{-1} for the α-helix, and at 1630 cm^{-1} for the β structure; for a deuterated polypeptide each of three frequencies is lower than what is given here by about 10 cm^{-1}). In principle, therefore, integrated intensities of the I and II bands should always be measured at each time to determine the number of peptide groups remained undeuterated. Sometimes, this is certainly found to be necessary. Quite often, however, the band widths of both amide I and II do not appreciably all over the reaction time (see Fig. 1, for example). In such a case, a simple measurement of the peak intensities can give an accurate enough kinetic data.

One of the merits of this method is its simplicity. In addition, the reliability of this method is rather easily judged by examining how good is the convergence of the several straight lines corresponding to the kinetic measurements of the slowly exchanging peptide NH groups under several different conditions (in temperature, pH, and/or LiCl concentration).

The peptide NH groups involved in the peptide-sidechain hydrogen-bonds (instead of the peptide-peptide hydrogen bonds) seem to escape completely to be counted in the proposed method.[1,3] By a proper adjustment of the pH and temperature of the solution, however, the number of the peptide NH groups in this category may also be determined by a similar kinetic method.[3] Sometimes, a further classification of the peptide-peptide hydrogen bonds may also be made by a kinetic study by means of an infrared absorption measurement similar to what is described above.

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