

been studying for these almost two decades. We presented the initial phase of our research at the US-Japan Seminar on self organization of proteins held at Cornell University in 1981. Since then several papers and reports were published in various publications.⁶⁻²⁰ This book is thus intended to provide the fundamentals of protein folding by presenting mainly a systematic explanation of the various researches done by Saitô and his collaborators.

1.2 Helix-Coil Transition in Polypeptide

1.2.1 α - and β -keratins

In 1951 Pauling, Corey *et al.*²¹ proposed new models of α - and β -keratin which are called α -helix and β -structure. Their proposed structures are shown in Figs. 1.2 and 1.3, that have turned out to be often found as building blocks of a protein, and are called secondary structures. Usually the right(left)-handed α -helix is observed for the poly-L(D) amino acid. The β -structure is a pleated

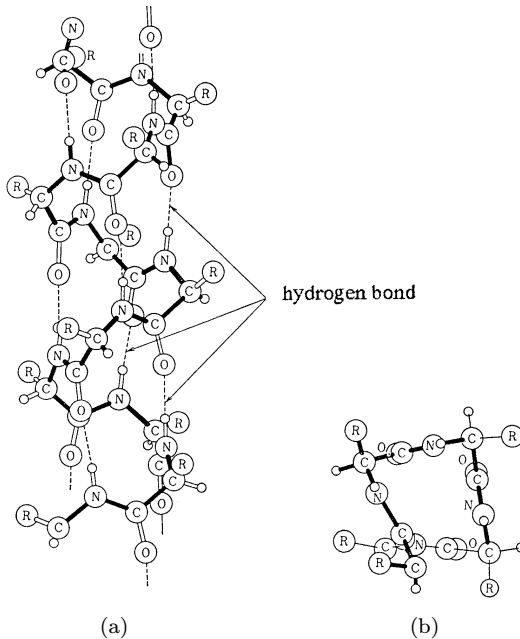


Fig. 1.2. Molecular models of α -helix.

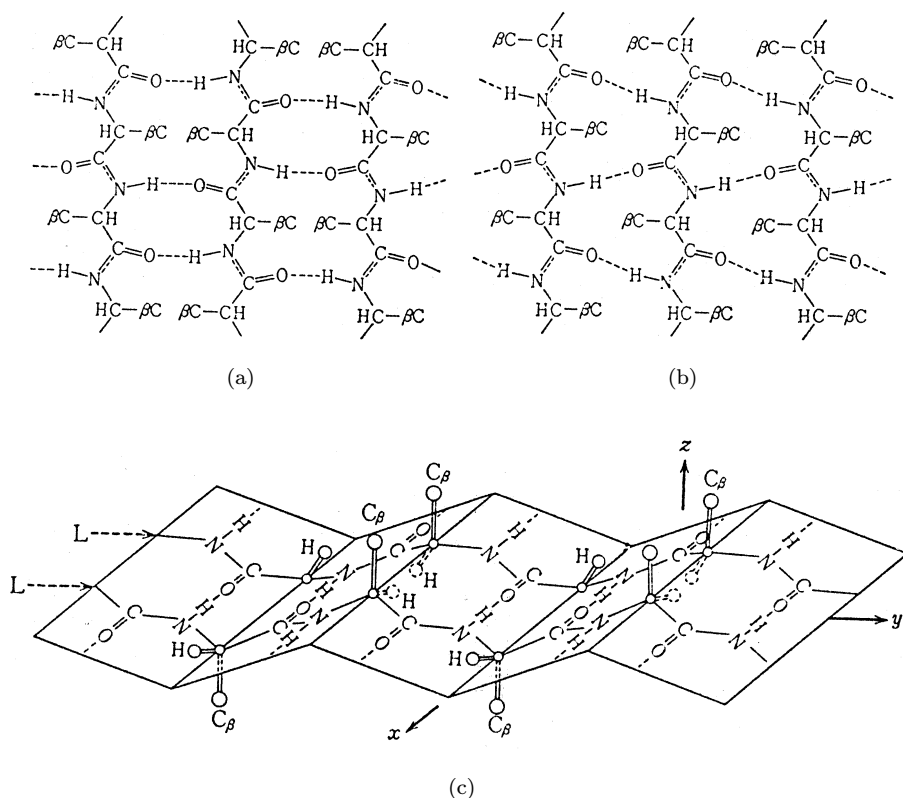


Fig. 1.3. Molecular model β -structures. (a), (c) Antiparallel β -structure. (b) Parallel β -structure.

sheet composed of β -strands in parallel or antiparallel arrangements. The guiding principles for constructing the model of α -keratin are as follows. (i) The peptide bond is planar, or in other words the angle ω is kept at 180° . The molecular parameters such as bond lengths and bond angles are the same as found in small molecules. (ii) The structure obtained by repeated arrangement of similar units is a helix and is stabilized by hydrogen bonds. (iii) The stable structure is searched for among the models satisfying (i) and (ii).

A polypeptide chain can take various conformations such as random coil, α -helix, β -structure, β -strand, etc. These conformations are characterized by the values of their dihedral angles ϕ and ψ . In particular, the values for α -helices

Table 1.2. Geometrical Factors in Polypeptides. 3_{10} -helix is included because it is sometimes found in real proteins.

Ordered Structure	ϕ (degree)	ψ (degree)	Pitch of helix (Å)	Number of amino acid residues per pitch
extended chain	+180	+180	7.3	2.00
right-handed α -helix	-57	-47	5.4	3.62
left-handed β -helix	+57	+47	5.4	3.62
3_{10} -helix	-4.9	-25	6.0	3.0
parallel β -sheet	-119	+113	6.5	2.0
anti-parallel β -sheet	-139	+135	7.0	2.0

and β -structures that correspond to the minimum values of their interaction energies are listed in Table 1.2. In this table the angles ϕ and ψ are taken clockwise with their origins at the extended conformation (see Fig. 1.1). The extended conformation is sometimes called β -strand. When two β -strands are put in parallel or in antiparallel position, they can form parallel or antiparallel β -structure, by slightly changing the dihedral angles. As explained later (Sec. 1.3), the secondary structures are formed first from a nascent polypeptide chain in random coil state while biosynthesis of protein. Their structures are supposed to be of standard form listed in Table 1.2. In the native proteins, however, the secondary structures are usually deformed from the standard forms. Kabsch and Sander²² thus presented DSSP (Define Secondary Structure of Proteins) program based on a set of simple and physically motivated criteria for secondary structure through a pattern-recognition process of hydrogen bonded and geometrical features extracted from X-ray coordinates. The DSSP program is available on the Web(<http://www.ddbj.nig.ac.jp/>). But it cannot necessarily reproduce the standard secondary structures described above (see Sec. 2.6).

1.2.2 Helix-coil transition

The stereoregular polypeptides composed exclusively of L-amino acids, such as polyglutamic acid (PGA) or polybenzyl-L-glutamate (PBLG), can be regarded as simple protein models. The solution properties such as viscosity $[\eta]$, ionization I and optical rotation $[\alpha]$, etc., of PGA and PBLG were investigated

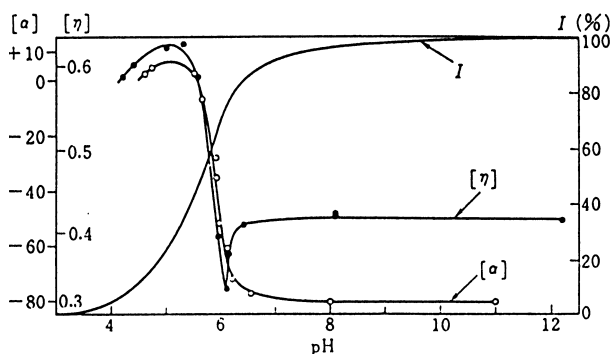


Fig. 1.4. Helix-coil transition in PGA (reproduced from Ref. 21 with permission).

by Doty and his collaborators.²³ They found that these molecules can take helical structures, identified as α -helices, under appropriate conditions. They further showed that the transition between helix and coil can be induced by changing the solvent conditions, as shown in Fig. 1.4 for PGA. In other words, α -helix first found in solid crystalline state is also stable in solution as a single molecule. We have employed the word transition, because one state changes into another reversibly under quite small changes of solvent conditions, similar to the phase transition between liquid and gas. However, in one-dimensional systems with finite range interaction no phase transition can be expected according to statistical mechanics.²⁴ The term transition used in statistical mechanics implies a change of state in a mathematically singular manner in the thermodynamic limit, i.e., in the limit of infinitely large number of particles and the volume with the density kept constant. In this sense, although the helix-coil transition in polypeptide is a sharp change of states, it is not, strictly speaking, a phase transition, because it does not have any mathematical singularity. Then, why does transition-like phenomenon occurs in polypeptide? Now consider a one-dimensional lattice gas with N -particles in M sites, where it is assumed that the nearest-neighbor interaction is repulsive and the second-neighbor interaction is zero, but it becomes sufficiently attractive to overcome the nearest-neighbor repulsive forces (Fig. 1.5), when a third particle comes in between. In this system when M (volume) is decreased, some particles have to make contact to form a doublet, resulting in an increase of the pressure, but upon further decrease of M a triplet of particles happen to be made without big increase of pressure. Consequently, the pressure increases steeply only in

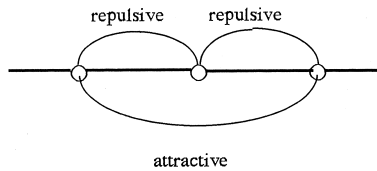


Fig. 1.5. Interactions among three particles.

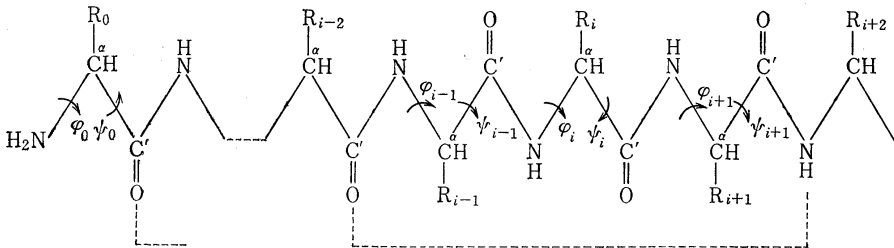


Fig. 1.6. Polypeptide chain.

the small range of M when it is decreased. This model is a lattice gas version of the helix-coil transition,²⁵ where forming an initial helix turn is unfavorable, since this conformation occupies a quite small region in the phase space. Once one helix turn is formed, further winding of a helix turn is easy thanks to the hydrogen bonding. In an α -helix of polypeptide a hydrogen bond is formed between the i th and the $(i + 4)$ th residues as can be seen from the model of α -helix shown in Fig. 1.6. Theories of helix-coil transition for homopolypeptide were developed by many authors (see Poland and Scheraga²⁶) especially by Hill,²⁷ Zimm and Bragg,²⁸ and Lifson and Roig.²⁹ In the next subsection we will present a theory for proteins regarded as heteropolypeptides, in order to apply it for the prediction of secondary structure. The theory reduces to the conventional helix-coil transition theory when applied to homopolypeptides (See Appendix A).

1.2.3 Theory of the formation of α -helices and β -strands in protein

For the formation of α -helices, β -strands, and further complex structures in proteins, we propose the island model. An island is defined as a part of the

chain which has a definite structure through local interactions among residues. For the present purpose we have only to consider α -helices, β -strands, and coil parts. It is assumed that there exists at least one residue in the coil state between an α -helix and a β -strand and there is no interaction between different islands. In the island model the formation of a structure proceeds first by the birth of embryos or kernels of islands and then by their growth through incorporating neighboring residues successively. The conformation of a polypeptide is described in terms of dihedral angles of two bonds from α -carbons. If a pair of dihedral angles around the bonds from the α -carbon C_i^α are specified, together with the internal rotations of the $(i-1)$ th, i th and $(i+1)$ th residues, and the structure of planar peptide bonds, they can determine the relative position between $O=C_{i-2}$ of the $(i-2)$ th residue and HNC_{i+2} of the $(i+2)$ th residue as shown in Fig. 1.6 by $---$. If the relative position becomes appropriate, a hydrogen bond for helix can be formed (see Fig. 1.2). In a β -strand the side chain attached to C_i^α of the i th residue comes close to the side chain of the $(i+2)$ th residue (see Fig. 1.3). The interaction is effective only when the dihedral angles around C_i^α , C_{i+1}^α , and C_{i+2}^α take the values appropriate for β -strands. The interaction for α helix or β -strand contribute to the statistical weights $w_2(i-2, i+2)$ and $u_2(i, i+2)$, respectively, which are different from pair to pair in the formulation to follow. By taking account of these statistical weights a statistical mechanical theory using a matrix formulation was developed by Wako *et al.*,³⁰ but here we present a formalism in the form of recurrence relations which is suitable for computer calculation.¹⁰

The partition function Z_n of a part of the protein from the 1st to the n th residue is written as:

$$Z_n = Z_n^{(\alpha)} + Z_n^{(\beta)} + Z_n^{(c)}, \quad (1.1)$$

where $Z_n^{(\alpha)}$, $Z_n^{(\beta)}$, and $Z_n^{(c)}$ are the partition functions with the n th residue in α -helix, β -strand, and coil, respectively. We have the recurrence relation:

$$Z_n^{(\alpha)} = \sum_{k=1}^n Z_{n-k}^{(c)} H(n-k+1, n), \quad Z_0^{(c)} = 1, \quad Z_0^{(\alpha)} = 0, \quad (1.2)$$

where $H(n - k + 1, n)$ implies the partition function of complete α -states from the $(n - k + 1)$ th to the n th residues, written as

$$\begin{aligned} H(n - k + 1, n) &= w_0(n - k + 1)w_1(n - k + 1, n - k + 2) \cdots w_{k-1}(n - k + 1, n) \\ &\quad \times w_0(n - k + 2)w_1(n - k + 2, n - k + 3) \cdots w_{k-2}(n - k + 2, n) \\ &\quad \times \cdots w_0(n - 1)w_1(n - 1, n) \end{aligned} \quad (1.3)$$

in terms of weight functions, $w_0(i)$ for single i th residue and $w_k(i, i + k)$ for a pair of the i th and the $(i + k)$ th residues. Similarly,

$$Z_n^{(\beta)} = \sum_{k=1}^n Z_{n-k}^{(c)} B(n - k + 1, n), \quad (1.4)$$

where $B(n - k + 1, n)$ is the partition function for complete β -states and can be written as Eq. (1.3) in terms of u for weight functions of β -states. Finally, we have

$$Z_n^{(c)} = \sum_{k=1}^{n-1} [Z_{n-k}^{(\alpha)} + Z_{n-k}^{(\beta)}] + 1, \quad (1.5)$$

and

$$Z_0^{(c)} = 1, \quad Z_1^{(c)} = 1, \quad Z_1^{(\alpha)} = w_0(1), \quad Z_1^{(\beta)} = u_0(1). \quad (1.6)$$

Furthermore, we assume that no interaction exists between the k th and the k' th residues in the same α or β part for $|k - k'| > p$ in α and $|k - k'| > p'$ in β . Thus,

$$w_k(n, n + k) = 1, \quad k > p, \quad (1.7)$$

$$u_k(n, n + k) = 1, \quad k > p' \quad (1.8)$$

and, consequently,

$$H(n - k + 1, n) = H(n - k + 1, n - 1)W(n - p, n), \quad k > p, \quad (1.9)$$

$$B(n - k + 1, n) = B(n - k + 1, n - 1)U(n - p', n), \quad k > p', \quad (1.10)$$

where

$$W(n-p, n) = w_p(n-p, n)w_{p-1}(n-p+1, n) \cdots w_1(n-1, n)w_0(n), \quad (1.11)$$

and a similar relation exists for $U(n-p', n)$.

In the following we shall discuss the α state only, because the results can easily be extended to the β state. Equations (1.1)–(1.6) can be employed successively for calculating $Z_n^{(\alpha)}$ for $n < p$. For $n > p$, we have

$$Z_n^{(\alpha)} = \sum_{k=p}^n Z_{n-k}^{(c)} H(n-k+1, n) + \sum_{k=1}^{p-1} Z_{n-k}^{(c)} H(n-k+1, n), \quad (1.12)$$

where the first term of rhs is

$$\begin{aligned} T^{(\alpha)}(n) &= \sum_{k=p}^n Z_{n-k}^{(c)} H(n-k+1, n) \\ &= \sum_{k=p}^n Z_{n-k}^{(c)} H(n-k+1, n-1)W(n-p, n) \\ &= [T^{(\alpha)}(n-1) + Z_{n-p}^{(c)} H(n-p+1, n-1)]W(n-p, n). \end{aligned} \quad (1.13)$$

In a similar way, the recurrence relations for $Z_n^{(\beta)}$ and $Z_n^{(c)}$ can be formulated. These recurrence relations can be used successively for calculating Z_N .

The partition function can also be written as

$$\begin{aligned} Z_N &= \sum_{k=1}^i \sum_{k'=1}^{N-i+1} Z_{i-k}^{(c)} H(i-k+1, i+k'-1) Z'_{N-k'+1}^{(c)} \\ &\quad + \sum_{k=1}^i \sum_{k'=1}^{N-i+1} Z_{i-k}^{(c)} B(i-k+1, i+k'-1) Z'_{N-i-k'+1}^{(c)} \\ &\quad + Z_i^{(c)} Z'_{N-i}^{(c)}, \end{aligned} \quad (1.14)$$

where $Z'_{N-i-k'+1}^{(c)}$ implies the partition function for the chain from the C terminus to the $(N-i-k'+1)$ th residue numbered reversely from the C terminus (which is the $(i+k')$ th residue from the N terminus) in the coil state.

The probability that the i th residue is in α is given by

$$p_i^{(\alpha)} = Z_N^{-1} \sum_k \sum_{k'} Z_{i-k}^{(c)} H(i-k+1, i+k'-1) Z'_{N-i-k'+1}^{(c)}. \quad (1.15)$$

Similarly, we have for β

$$p_i^{(\beta)} = Z_N^{-1} \sum_k \sum_{k'} Z_{i-k}^{(c)} B(i-k+1, i+k'-1) Z'_{N-i-k'+1}^{(c)}. \quad (1.16)$$

1.2.4 Prediction of α -helices and β -strands in a protein

In order to calculate the probabilities $p^{(\alpha)}$ and $p^{(\beta)}$ we have to determine the values of statistical weights, w 's and u 's. They are to be obtained, in principle, from physicochemical calculations, but now we take another method following Wako *et al.*³⁰ to determine them so that they can recover the secondary structures of known protein structures. To do this we introduce the objective function F defined by

$$F = \sum_P \left[\sum_{j \in \alpha} \{(1 - p_j^{(\alpha)})^2 + (p_j^{(\beta)})^2 + (p_j^{(c)})^2\} \right. \\ \left. + \sum_{j \in \beta} \{(p_j^{(\alpha)})^2 + (1 - p_j^{(\beta)})^2 + (p_j^{(c)})^2\} \right. \\ \left. + \sum_{j \in c} \{(p_j^{(\alpha)})^2 + (p_j^{(\beta)})^2 + (1 - p_j^{(c)})^2\} \right], \quad (1.17)$$

where the first sum of this equation is taken over all the referring proteins of number N_P . When $F = 0$ is achieved, the prediction for the referring proteins becomes perfect. For our present purpose it would be sufficient to take $p = 4$ (see Appendix A) and $p' = 2$. Then the number of necessary parameters are $20 + 20 \times 20 \times 4 = 1620$ for α -helix and $20 + 20 \times 20 \times 2 = 820$ for β -strand. If we take $p = p' = 4$, we have to determine 3240 parameters by minimizing the objective function F . In the following section, we will present both the cases to see how the determination of the necessary parameters proceeds.

1.2.5 Some preliminary results for the secondary structure prediction

The minimization of the objective function has not yet reached the level as we have wished, but the preliminary results obtained up to now should be of some interest for future developments.

We optimized the weight parameters using 80 proteins (not listed here) which lack sequential homology among them, and then estimated the prediction accuracies for 13 proteins (shown in Table 1.3, column (a)) other than the 80 reference proteins. Firstly, we minimized the objective function F by changing all of 3240 parameters. The estimation results for the 13 proteins indicates that the overall accuracy (= total number of correctly predicted residues (α , β and coil) in 13 proteins/total number of residues of the 13 proteins) is 69.6%, while the accuracies of α -helices ($ACR(\alpha)$) and β -strands ($ACR(\beta)$) (= total number of correctly predicted residues in α -helices (or β -strands) of 13

Table 1.3. Prediction accuracy for 13 proteins for estimation. Column (a), all of 3240 parameters were optimized. Column (b), the values of the parameters for $k = 3$ and 4 in β -strands were set at 1.

PDB-ID	No. of Residues	Accuracy	
		(a)	(b)
1ACX	108	59.259	62.037
1CTE	68	64.706	52.941
1LH1	153	84.314	64.967
1UBQ	76	67.105	77.632
2ALP	198	53.030	51.010
2CDV	107	75.701	77.570
2CI2	65	69.231	58.462
2WRP	104	57.692	56.731
4RHV2	255	69.020	65.882
3CLN	143	67.832	62.937
4FXN	138	91.304	89.855
5LYZ	129	68.217	64.341
7RSA	124	74.194	72.581
Total	1668	69.425	67.626

proteins/number of residues observed in α -helices (or β -strands) of the 13 proteins) are 66.3% and 46.9%, respectively. These numbers are more important than the 69.6%, because the latter includes a big contribution of the correctly predicted coil states.

The reasons for the poor accuracy, especially in β -strands, are as follows:

- (1) 457 pairs among 3240 pairs of the 13 proteins are not observed in the secondary structures of the 80 proteins. Thus the parameters for the missing pairs cannot be optimized properly.
- (2) The distance of the interactions was taken $k \leq 4$ in case of β -strands as well as in case of α -helices, but the statistical weights of interacting pairs of the i th and the $(i \pm k)$ th residues ($k = 3, 4$) may not be significantly different from 1 in β -strands. Thus, the parameters for these pairs may be estimated artificially and erroneously during the optimization process.

The number of correctly predicted residues depends on the degree of the optimization of the parameters corresponding to the pairs necessary for prediction. We hoped that the above mentioned accuracy would be improved by assuming $k \leq 2$ for β -strands and by reducing the number of amino acid pairs in the proteins for optimization. Thus, we optimized again the weight parameters, assuming $k \leq 4$ for α -helices and $k \leq 2$ for β -strands. We assigned unity as the value of the parameters for $k = 3$ and 4 in β -strands and performed the optimization with these parameters fixed. As Table 1.3(b) shows, the prediction accuracies for the 13 proteins fall between 51% and 90%, and the average accuracy of the proteins for estimation is 68.1%, with $\text{ACR}(\alpha) = 65.4\%$, and $\text{ACR}(\beta) = 45.9\%$. These values are lower than those of Table 1.3(a), where all of 3240 parameters are optimized. The prediction accuracy of the 80 proteins for the parameter optimization is only 78.2% in (b), but it is 79.2% in (a).

This suggests two features. First, the parameters, 1620 for α -helices and 820 for β -strands, have not yet been optimized sufficiently. As Table 1.4 shows, the number of the residues predicted correctly in the 80 proteins for the parameter optimization increases as the optimization process progresses. The prediction accuracy of β -strands was remarkably improved. Second, a β -strand usually makes a β -sheet with neighboring β -strands. This fact may introduce some indirect interactions between $k = 3$ and 4 pairs, resulting better prediction accuracy than the case $k \leq 2$. Or, speaking physically, since the structures are sometimes susceptible to slight changes while making a β -sheet

Table 1.4. Improvement of prediction accuracy during the process of the parameter optimization. The values of the parameters for $k = 3$ and 4 in β -strands are set at 1. These results show the numbers of the residues correctly predicted and those incorrectly predicted for the 80 proteins for the parameter optimization. NR is the total number of residues of these 80 proteins. NHH is the number of the residues correctly predicted as α -helix, NHE is the number of the residues incorrectly predicted as β -strand, though they are actually in α -helix and NCH is the number of the residues incorrectly predicted as α -helix, though they are actually in coil. The other notations are analogous to these. ACR is the prediction accuracy. As the parameter optimization proceeded, as indicated from the 2nd to 4th lines, the prediction accuracy, especially of β -strands is found to have improved.

NR	NHH	NEH	NCH	NHE	NEE	NCE	NHC	NEC	NCC	ACR
11778	2035	67	314	30	1771	467	617	957	5520	79.182
11778	2048	91	352	60	1351	549	574	1353	5400	74.707
11778	2053	98	335	58	1527	575	571	1170	5391	76.167
11778	2082	83	347	42	1709	525	558	1003	5429	78.282

(Sec. 1.2.1), folding proceeds with the standard β -strand not necessarily the same as in the crystalline state, but take the native structures after making a β -sheet. Similar features like this are often observed in the actual process of folding (see Secs. 2.2.3 and 2.6). The secondary structures are usually more or less deformed at the final stage of folding. Furthermore, antiparallel β -sheets must be determined through checking whether the neighboring two β -strands have the ability to make an antiparallel β -sheet (see Appendix D). Again they are usually deformed. Thus we reach the conclusion that the numerical values of the statistical weights, w 's and u 's are for standard structures, and for this purpose one has to prepare referring proteins having standard secondary structures. Practically it is difficult, if not impossible. This may be a possible reason for the unsatisfactory prediction accuracy of the secondary structures, especially of β -strands. This consideration holds for empirical methods of prediction to be discussed in Sec. 4.5.1.

The precision of order ca.70% in Table 1.3 seems to be the upper limit of any method for secondary structure.

We should note that various methods of the secondary structure prediction have been developed mostly by the homologies between the primary structures and by the statistical inference based on protein data base, and their prediction accuracies have reached about 70%.^{112,113} That is, our method gives results comparable to them (see Sec. 4.5.1). In this connection, we refer to the

proposal of Rost, Sander, and Schneider.¹⁶¹ They showed that considerable variation in the position and length of secondary structure segments can be accommodated within the same three-dimensional structure. Thus, the goal of the prediction accuracy, they say, can be reduced to some extent, and a new measure of segment overlap is introduced to compromise between permissiveness and precision. But it is unknown whether or not the accommodated secondary structures are consistent with the folding mechanism to be discussed in Sec. 1.3. We think that more effort is required to improve the accuracy of secondary structure prediction.

1.2.6 Stability of α -helix

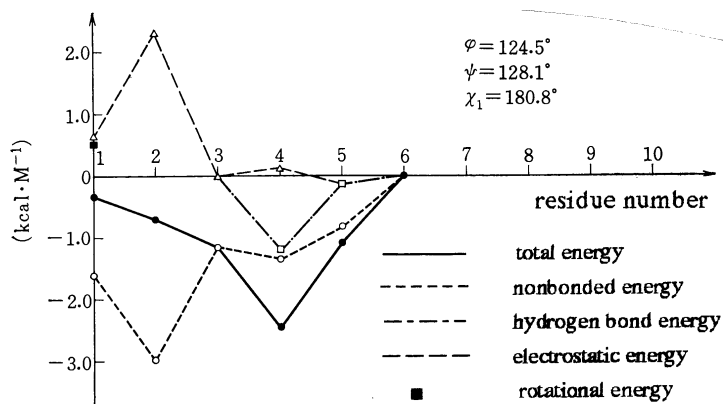
The conformational energy of an α -helix is composed of several energies; for example, rotational energies around the dihedral angles, nonbonded interactions between the atoms in the chain, electrostatic energies and hydrogen bonds. Among them the hydrogen bonds between two amino acid residues that are 3 residues apart (in other words at a medium distance) in the main chain play the decisive role for the formation of the helix as can be seen in the theories of helix-coil transition. The contribution of various energies mentioned above in the conformation of poly-L-alanine in the crystalline state was calculated by Kosuge *et al.*³¹ improving the result of Ooi *et al.*³² Table 1.5 gives their results and shows that the largest contribution comes from the nonbonded interactions among mostly nearest neighbors which cannot, by themselves, hold stably the helical structure. On the other hand, the contribution from the hydrogen bonds is smaller but is most important for the stability as explained below. In the case of helix formation the hydrogen bond is directional, short-range and

Table 1.5. Conformational energy per residue of the α -helical structure of poly(L-alanine).

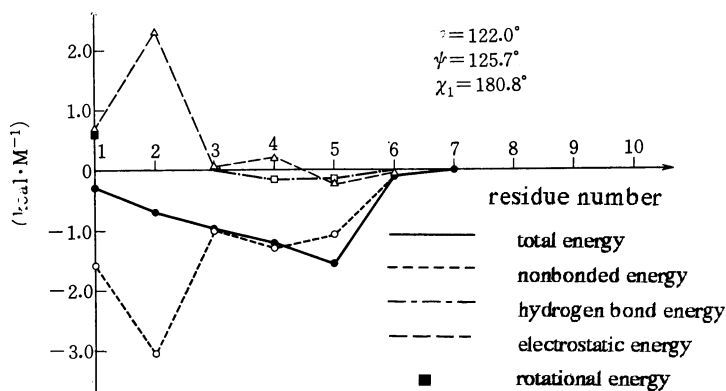
	Energy (kcal/mol-res)	
	Ooi <i>et al.</i>	Kosuge <i>et al.</i>
Rotational	0.49	0.58
Nonbonded	-5.99	-7.15
Electrostatic	-1.10	2.60
Hydrogen bond	-1.74	-1.02
Total	-8.34	-5.00

medium-distance interaction in our terminology to be defined in Sec. 1.3.3. It is noted that the hydrogen bond energy can easily disappear, while other interactions do not change significantly, when the helical structure is slightly deformed, as shown in Fig. 1.7.³¹

This fact implies that the largest nonbonded interactions, if alone, cannot hold the helical structure. The energy function of a hydrogen bond has a



(a)



(b)

Fig. 1.7. Distribution of energies among residues (reproduced from Ref. 31 with permission). (a) α -helix. (b) Slightly deformed α -helix.

sharp minimum with respect to the atomic distance and the bond is thus easily broken by a slight deformation of the helical structure. Even if their energy is smaller than the nonbonded energy, hydrogen bonds are essential for keeping the α -helix, and bring about the stability. Although the above calculations are performed in vacuum, recent elaborate calculations of interaction energies in α -helix by taking account of solvent effect by Yang and Honig³³ show that the numerical values are not qualitatively different from those in vacuum and yet the largest contribution comes from nonbonded and hydrophobic interactions in agreement with Kosuge *et al.* and Ooi *et al.* However Yang and Honig do not calculate the energies in deformed states (See Sec. 4.6 for the effect of water). We can conclude as well that the hydrogen bonds are the main factor for the stability of the α -helical structure in the sense mentioned above, provided that the hydrogen bond has short-range energy.

1.3 Some Aspects of Protein Folding

1.3.1 *Reversible denaturation and renaturation: Anfinsen's dogma*

The conformation of a globular protein in solution at ordinary temperatures is quite complicated without any geometrical symmetry, but it is an ordered state in the sense that it has biological activity. It is supposed to be almost the same as that of crystalline state as already mentioned. This complicated conformation of a single protein molecule is destroyed upon increasing the temperature or by the addition of appropriate chemical agents, as revealed by the loss of its activity and the change of the physical quantities such as optical properties, solution properties, and so on. Once the complicated native structures having biological activity is lost, it would be natural to suppose that the native structure could hardly be restored. Nevertheless, some pioneers such as Anson and Mirsky recognized as early as in 1925 that this was not always the case. Convincing and beautiful experiments were carried out by Anfinsen *et al.*^{34,35} for ribonuclease and, independently, by Isemura *et al.*^{36,37} for takamylase around 1960. Their surprising experimental facts demonstrate clearly the reversible nature of denaturation and renaturation. The denatured proteins can recover the biological activities and their complicated conformations, when their respective conditions of the solution are restored. Isemura, Takagi and others,^{37,38} furthermore, were able to obtain a crystalline state from the