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Mechanism of protein folding

II. Lysozyme and phospholipase

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Refolding of hen egg-white lysozyme assuming the formation of secondary structures (α-helices and β-sheets) is carried out by the method presented in the previous paper (N. Saitô et al., Proteins; Struct. Funct. Genet. 3 (1988) 199–208). To do this, the hydrophobic interactions between the hydrophobic residues which are located at the key positions for folding and can be identified without te knowledge of the native structure, and the nonbonded interactions between every pair of atoms (except hydrogen) or groups are introduced successively from short- to medium-distance pairs. The search for the energy minimum by these interactions can afford a conformation of especially the mutual arrangements between neighboring secondary structures. When these local structures are accomplished, some of the long-distance amino-acid pairs come close together and then the possible interactions (hydrophobic, nonbonded) are introduced. The three-dimensional structure of lysozyme thus obtained is shown to have locally correct arrangements of the secondary structures, but mutual relations between long-distance parts of the chain are not similar to the native structure. The introduction of disulfide bonds between appropriate cysteine residues is necessary to reach the native structure. The choice of cysteine pairs for disulfide bonding is made by the criterion given in the paper to follow (K. Watanabe, A. Nakamura, Y. Fukuda and N. Saitô, Biophys. Chem. 40 (1991) 293). The same treatment is applied to bovine pancreatic phospholipase with 7 disulfide bonds. The formation of the antiparallel β-structures from neighboring β-strands and the problem of the folding order are also discussed.

1. Introduction

In the previous paper [1] (hereafter referred to as I) the mechanism of protein folding was proposed and applied to refolding of myoglobin which has α-helices only as secondary structures. This new mechanism will be approved by the success in refolding various proteins of known native structures. Thus in the present paper we discuss lysozyme and phospholipase of $\alpha+\beta$ type having α-helices and β-sheets and many disulfide bonds. Figure 1 shows the native structure of lysozyme, where α-helices and β-strands are numbered from N-terminus. The symbols (■) and (□) indicate the pairs of amino acid residues with the distance between the α-carbons less than 13 Å, and (■) and (×) indicate the hydrophobic pairs with the distance between the α-carbons less than and more than 13 Å apart respectively. Here we take Trp, Ile, Leu, Phe, Val, Met as hydrophobic amino acid residues. Three consecutive β-strands β_3, β_4, and β_5 form a β-sheet, leaving β_1, and β_2 as strands. The formation of this structure will be discussed in Section 4. the key hydrophobic pairs which

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bind neighboring secondary structures (α-helices, β-strands and β-sheet) are circled as (○). It can also be recognized in Fig. 1 that the circling can be done without the knowledge of the native structure provided that the secondary structures are formed (see Section 2), and thus the circled pairs are specified. When the circled pairs of hydrophobic residues are bound the energy decreases. Thus folding of $\alpha_1 \sim \alpha_6$-helices and β-structures (β_1, β_2 and a β-sheet) into the tertiary structure is carried out through searching for the conformation of minimum energy, thereby considering long-range hydrophobic interactions between short- or medium-distance pairs, which are specific in the meaning mentioned above. The nonbonded interactions between every pair of atoms (except H) are also considered from short- to medium- and long-distance ones. The distinction between the two words distance and range was made in I. For example, the long distance interaction is used between the two amino acid residues separated by many residues on the chain, while the long-range interaction is the one which is effective over a wide range in the usual three-dimensional space like hydrophobic interaction. When the local conformations between neighboring secondary structures are accomplished, the long distance pairs of amino acid residues come close together, and the possible interactions between them are introduced. In order to reach the native structure, we have to consider disulfide bonding between relevant cysteine residues,

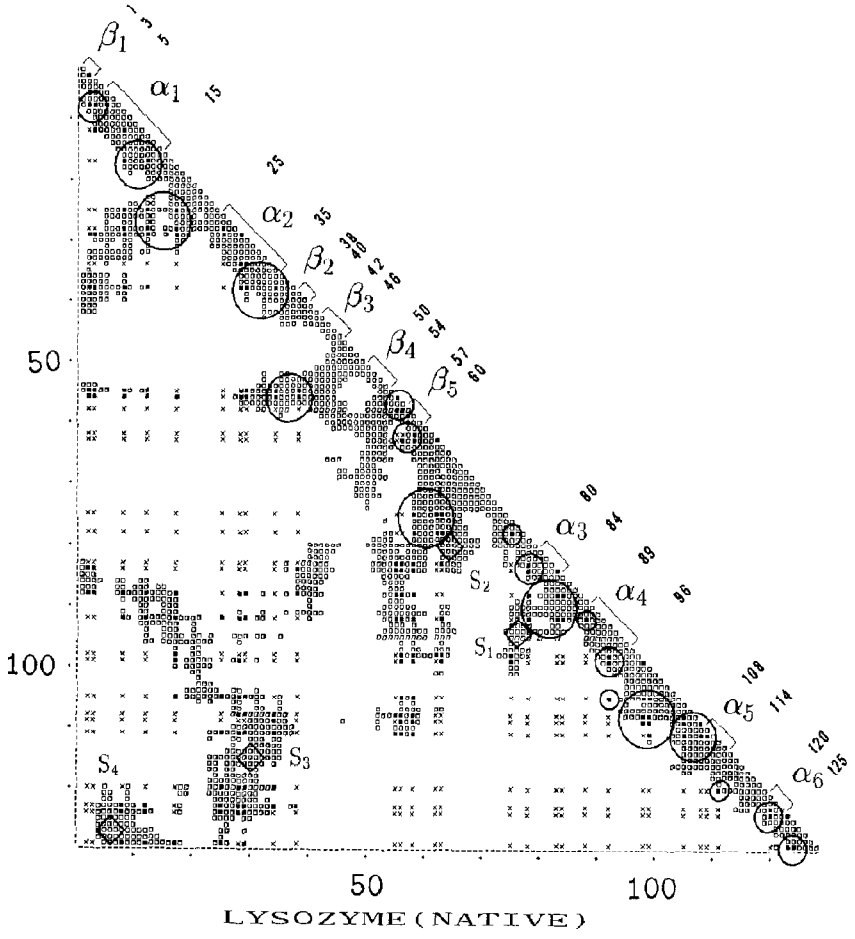


Fig. 1. Distance map of the native structure of lysozyme.

thereby selecting the proper pairs of cysteines The method of selection will be discussed in the paper to follow, which is referred to as III [2], but in the present paper we introduce the cysteine pairs in the native structure by anticipating the results in III. Figures 6 and 7 show the result for lysozyme, which can reproduce the native structure quite well. The similar treatment is performed for phospholipase in Section 3. The possible mechanism of the formation of antiparallel β-structures is discussed in Section 4. Section 5 is devoted to the discussion especially focussed on the order of folding.

2. Refolding of lysozyme

The method of refolding lysozyme is almost the same as with myoglobin. However, for the convenience of the independent reader, it may be useful to describe again the main ideas for protein folding. From the static point of view, the three-dimensional conformation of a protein is governed by thermodynamics and it is regarded as the state of the least free energy in "phase space" (for the meaning of "" see below). On the other hand from the dynamical point of view the folding process is quite rapid [3]. Consequently in order to reconcile the two aspects of thermodynamics and rapid dynamical process, it is required that the "phase space" itinerated by a point representing the conformation of a protein is not the whole phase space, implying that the state of the lowest free energy is attained not through random search but along some definite pathway, and thermodynamical consideration applies in this restricted space. We can assume that the folding from a denatured state proceeds along the following steps: (i) the formation of α-helices and β-strands, (ii) the formation of antiparallel β-sheets from neighboring β-strands and, (iii) packing of secondary structures (α-helices, β-strands and β-sheets) into a tertiary structure. The steps (i) and (ii) will be completed immediately, and thus the rate determining process of folding is attributed to the step (iii). Rapid folding must proceed without trial and error, or without repeated folding and unfolding of various wrong intermediate structures. To do this a particular nature of interaction is required. This is the long-range interaction between specified residues. The short-range interaction such as the Lennard–Jones potential is not effective, because a change of dihedral angles gives rise to the alteration of the arrangement of small-distance atoms and a pair of certain atoms will be separated, but another pair may happen to come close together, keeping the interaction energy almost unchanged, thus yielding multiple minima. On the other hand, the long-range interaction, if it is effective not on all the pairs of atoms but only on selected ones, will be weakened or strengthened by a change of the dihedral angles of the residues between the pair, which gives rise to a reasonable amount of energy change. Consequently the formation of the folding pathway is possible by a long-range specific interaction with the property that the pairs of the interaction can be specified without ambiguity. The long-range interaction required for this purpose is the hydrophobic interaction, acting between short- or medium-distance hydrophobic residues, and the so-called island model [4,5] is suitable for the specificity of the interaction. In this model, when hydrophobic interactions are assumed to play the main role, they are first introduced between short-distance residues at the beginning of folding and then extended to medium- and long-distance ones. The only thing to do is to confirm that the pairs of hydrophobic residues at the short and medium distance, after the formation of secondary structure, are all bound just as required for the validity of the island model.

Figure 1 is the distance map of the native structure of lysozyme with cutoff distance 13Å. As we have mentioned in Section 1, the pairs of hydrophobic residues encircled by (○) are situated at the key positions for combining neighboring secondary structures (6 α-helices, a β-sheet and two β-strands). Consequently the same procedure as in the case of myoglobin can be applied for the formation of the tertiary structure. In other words, long-range hydrophobic interactions and non-bonded interactions are considered, and the rigid side-chain conformations are assumed as described in I, Section 3. Then we search for the conformation of minimum energy starting from an

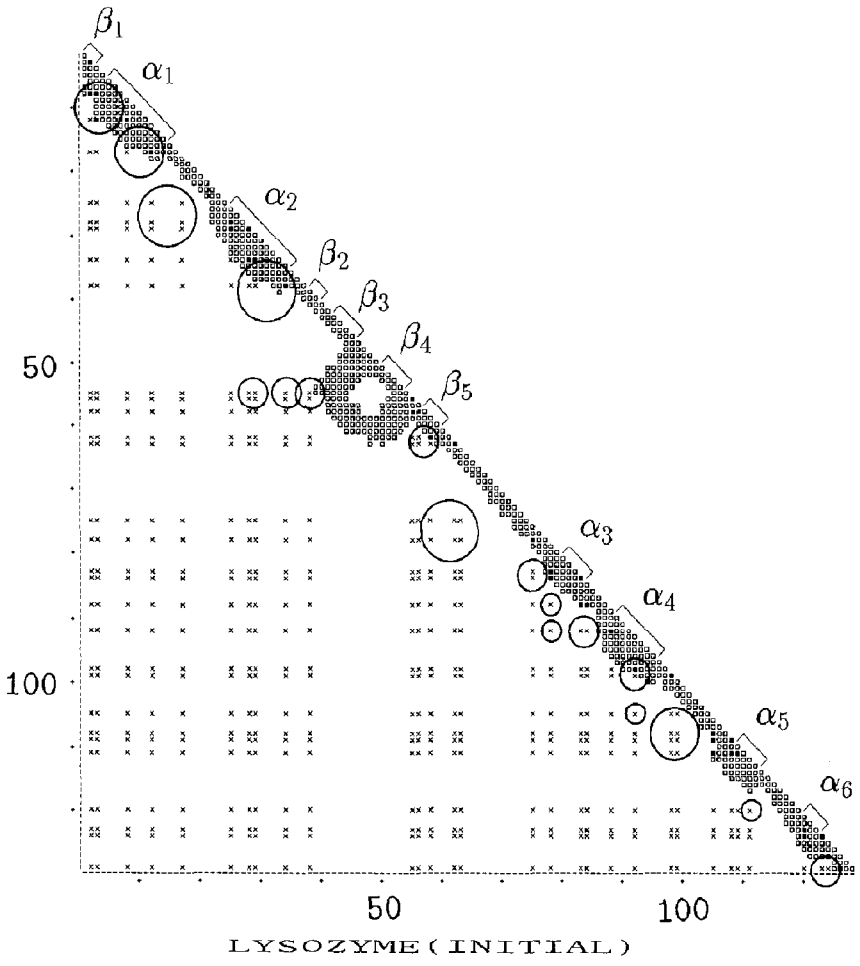


Fig. 2. Initial structure of lysozyme which was taken as a starting point.

extended conformation other than the parts of α-helices and β-sheets having the same structures as the native ones (Fig. 2). By looking at the distribution of hydrophobic pairs marked by (×) in Fig. 2, we can circle those pairs situated near the diagonal, or, the short-distance pairs. We do not have to consider the hydrophobic pairs within the same secondary structures, and have only to pick up a small number of those pairs which are necessary and sufficient to bind the neighboring secondary structures. This can be done without having a priori knowledge of the tertiary structure, but the encircled pairs will be effectively the same with the native structure. We do not worry about how many pairs are necessary and sufficient, because some medium-distance pairs will be unbound by the induced steric effects of the structure constructed by the short-distance pairs only. By changing the dihedral angles of the residues of the part between the two neighboring secondary structures we search for the state of minimum energy which is achieved by the formation of hydrophobic interactions of encircled pairs. The detailed procedure is described in I and the Introduction of the present paper.

It should be noted that in the present calculation we do not consider the chain entropy which decreases gradually as the formation of tertiary structure proceeds. We calculate only the energy part of the free energy, G, although in a hydrophobic interaction as described in I, the entropy of the water structure is taken into account. In the

island model, folding proceeds with the formation of local structures by virtue of the interactions between short- or medium-distance paris which are composed of long-range hydrophobic interaction and short-range Lennard–Jones potentials accompanied by the decrease of chain entropy ($\Delta S < 0$). Thus the decrease of energy is compensated partly by the increase of the entropy contribution of the free energy ($-T\Delta S > 0$). Here the decrease of free energy is always guaranteed ($\Delta G = \Delta H - T\Delta S < 0$), because in the island model the random coil region which is incorporated into the island already established is small and thus the decrease of chain entropy is also small. Otherwise the formation of the tertiary structure will not take place. A negative free energy of reaction, $\Delta G < 0$, is achieved provided that $\Delta H < 0$ and T is low. Therefore the energy calculation is supposed sufficient for the present purpose to construct the tertiary structure. Above consideration also justifies the island model for protein folding.

Figure 3 is a conformation obtained by the calculation described above. The local conformation is similar with the native one, but the arrangements of the long-distant pairs are quite unsatisfactory. Obviously we must take account of the disulfide bonds. The disulfide bonds are marked by (◇) in Fig. 1. They are denoted as S_1(Cys 76–Cys 94), S_2(Cys 64–Cys 80), S_3(Cys 30–Cys 115) and S_4(Cys 5–Cys 127). By looking at Figure 3 for the refolded structure, the distances of α-

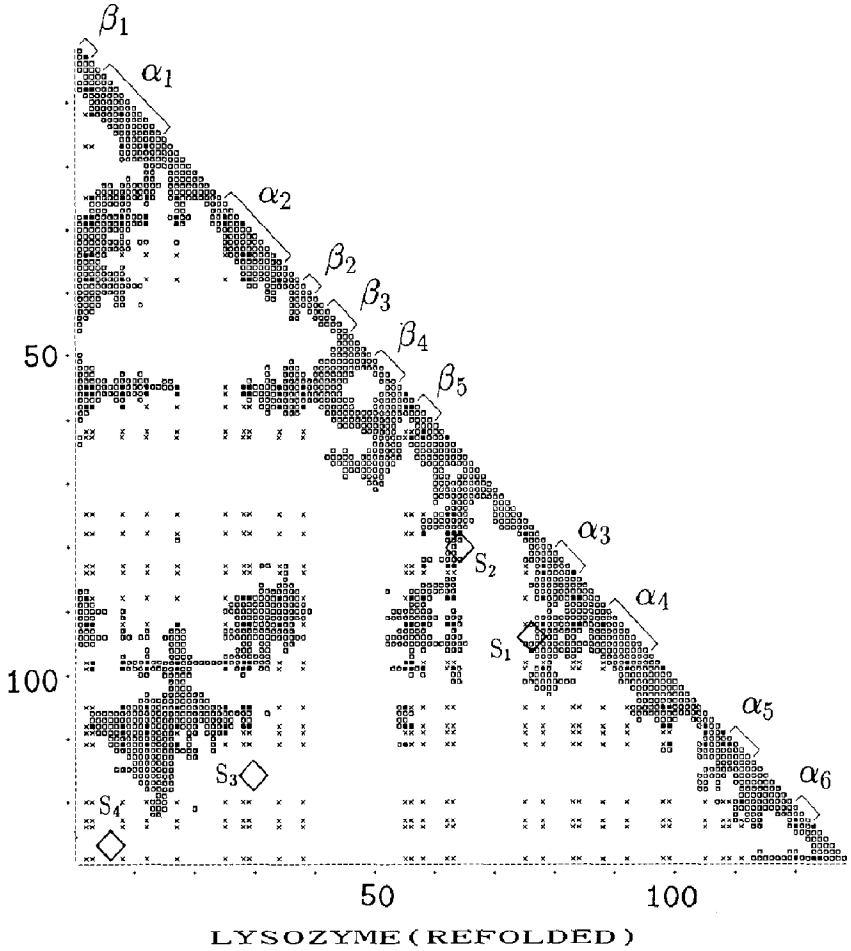


Fig. 3. Refolded structure of lysozyme without disulfide bond formation.

carbons for the S_1 and S_2 pairs are nearly 13Å, but the distances of α-carbons for the S_3 and S_4 pairs are not. The formation of a disulfide bond is a process of oxidation. Two SH groups are required to come close to each other and some oxidant also comes in between them. Thus similarly to the case of hydrophobic interaction, the interaction between two S atoms is effectively long-range. We may assume the energy of interaction to be of the form

$$E = \begin{cases} 20 & r < 3.2 \\ -10\left(1-(r-4.2)^2\right) & 3.2 \le r < 5.0 \\ -3.6\left(1-(r-5.0)^2/25\right) & 5.0 \le r < 10.0 \\ 0 & 10.0 \le r \end{cases} \quad (1)$$

which is composed of two parts: a short-range chemical bond (S–S) [6] and a long tail representing long-range interaction (Fig. 4). In the present calculation, the interaction energy (1) is introduced between the spheres representing the side chain of cysteine in accordance with the model employed here for amino acid residues. Furthermore in order to achieve a disulfide bonding an appropriate geometrical arrangement of –S–H groups is required because of the directional nature of chemical bonding besides the long-range property mentioned above. As the result the disulfide bonds S_1 and S_2 can be formed instead of

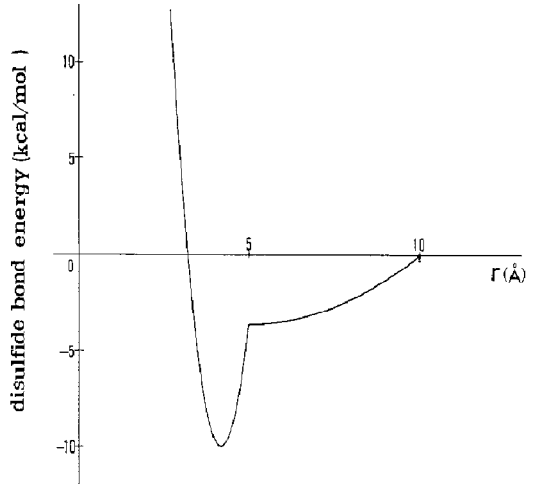


Fig. 4. S–S interaction in disulfide bonding.

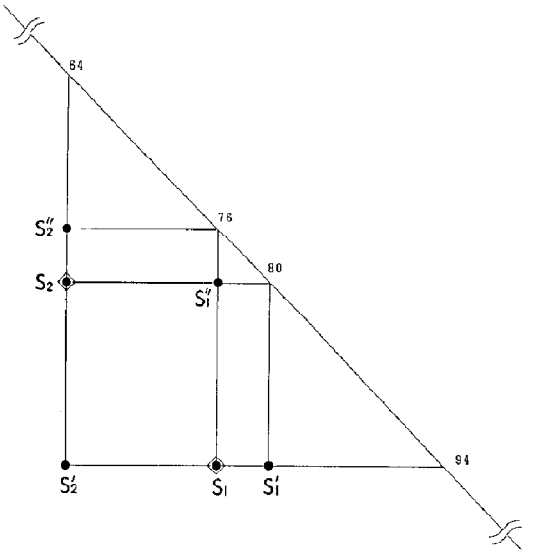


Fig. 5. Cys–Cys pairs in lysozyme.

S_2''(Cys 64–Cys 76) and S_2'(Cys 64–Cys 94) or other combinations (S_1', S_1'') (see Fig. 5), although the cysteines of each of these 6 pairs are close, respectively. Detailed discussions of the mechanism of selection among possible pairings of cysteines will be discussed in terms of geometric relations in III of this series [2]), because it requires a little lengthy description, digressing from the main topics. Here we employ the results of III, and introduce the interaction represented by eq. (1) between the cysteines of pairs S_1 and S_2, which make disulfide bonds in the native structure. The reasonable method to select S_1 and S_2 is to consider the test of the lampshade described in III. In this way, when S_1 and S_2 are formed the short-distance local structure becomes more and more close to the native structure, with the result allowing the formation of S_3 and S_4. The results are shown in Fig. 6 for the distance map and in Fig. 7 for the backbone structure of the main chain. One may say that they are satisfactory compared with the native structure. However, the pairs S_3 and S_4 cannot approach each other closer than in the conformation represented by Fig. 6 where the S_3 and S_4 are not yet bonded, even if we proceed further computation. This is supposed owing to the rigid side chains assumed in the above considerations. At the final stage of folding, the conformation becomes more and more closely packed

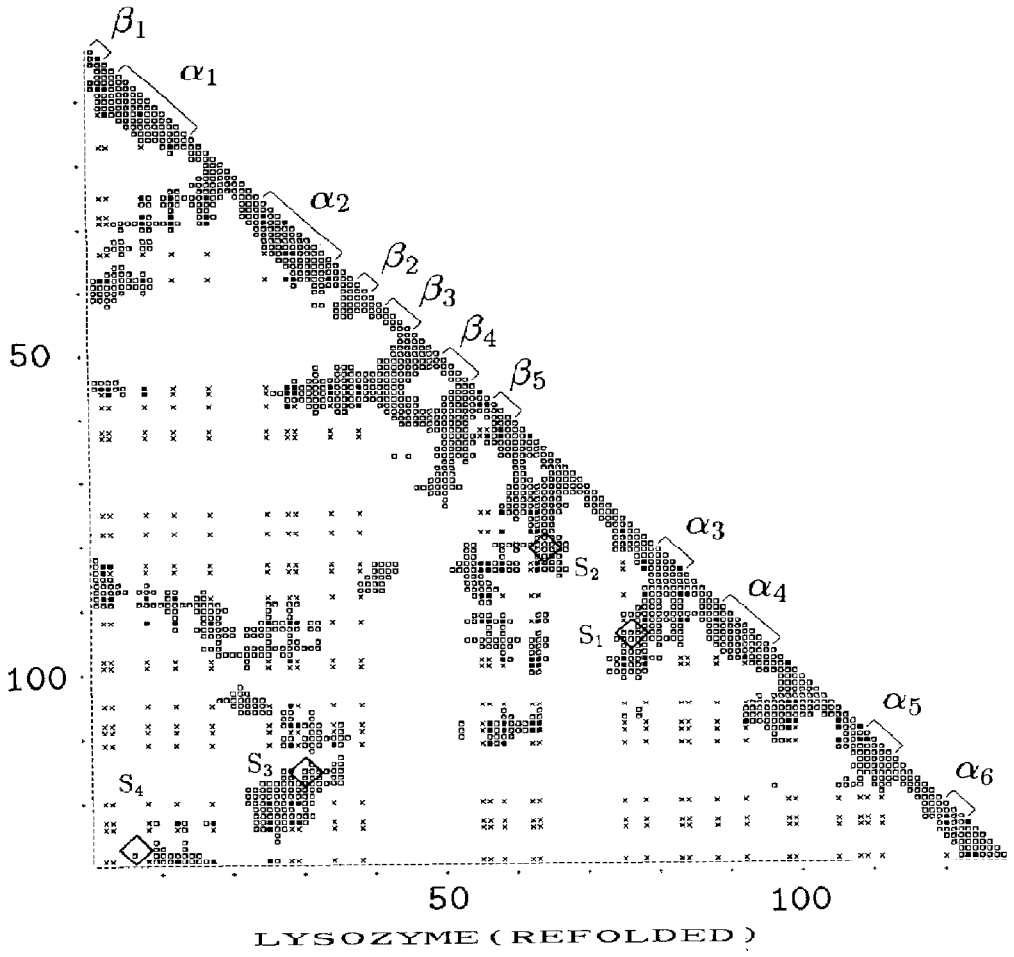


Fig. 6. Refolded structure of lysozyme with the disulfide bonds S_1 and S_2 (2900th step).

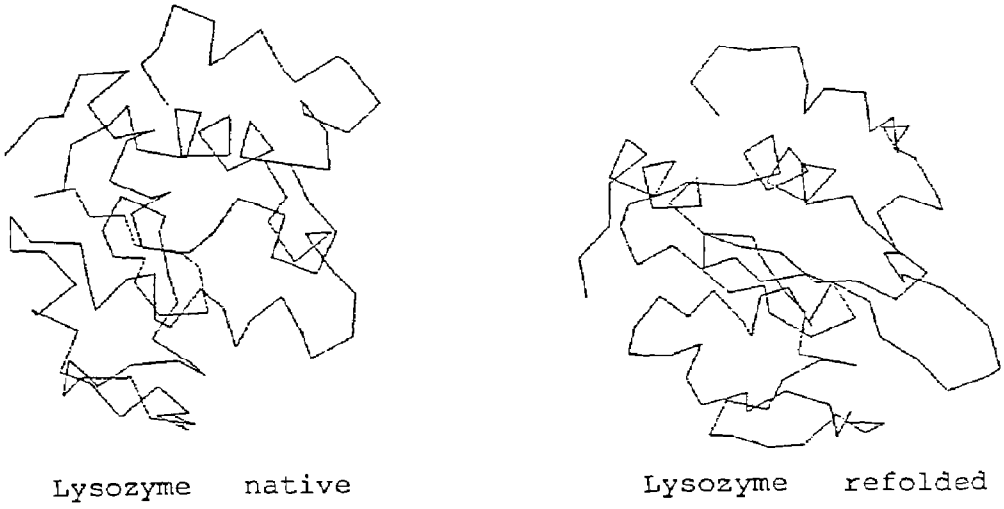


Fig. 7. Backbone structure of lysozyme for native (Fig. 1) and refolded (Fig. 6) structures.

and consequently flexibility of side chains must be considered to reach the native structure. This is also the case in phospholipase.

3. Refolding of phospholipase

Bovine pancreatic phospholipase has 5 α-helices and 2 β-strands and 7 disulfide bonds. The native structure is illustrated in Fig. 8, with cutoff distance 13 Å using the same symbols as in Fig. 1. The hydrophobic residues used in Fig. 8 are Trp, Ile, Leu, Val, Phe, Met, and Cys. We consider here Cys also as hydrophobic (see Section 4), because it is important for the formation of disulfide bonds. Two β-strands form an antiparallel β-structure, which is discussed in Section 4 together with the β-structures in lysozyme. The seven disulfide bonds are named as S_1(Cys 84–Cys 96), S_2(Cys 61–Cys 91), S_3(Cys 51–Cys 98), S_4(Cys 44–Cys 105), S_5(Cys 29–Cys 45), S_6(Cys 27–Cys 123) and S_7(Cys 11–Cys 77), respectively. According, to the crystal data, α_3 helix is from 39 to 58 and α_4 is from 58 to 66. This implies that the formation of a long $\alpha_3 + \alpha_4$ helix is prevented by the non-helical conformation of the dihedral angles of the 58th amino acid residue. Thus we start from the extended conformation composed of 5 helices (with long $\alpha_3 + \alpha_4$ helix) and one antiparallel β-structure as shown in Fig. 9, and pack these secondary structures into a tertiary structure by changing the dihedral angles of the parts other than the sec-

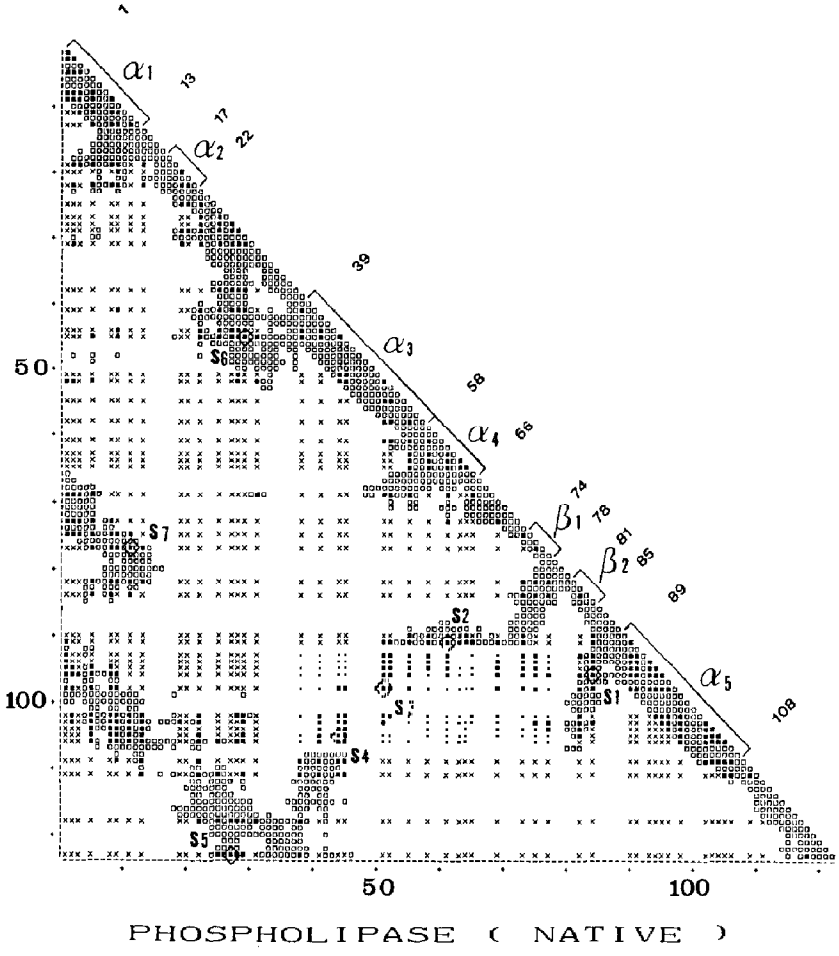


Fig. 8. Distance map of the native structure of phospholipase.

ondary structures, with consideration of the hydrophobic pairs (which have been encircled in Fig. 9) without prior knowledge of the tertiary structure at the initial stage. We take account of other hydrophobic pairs when they come close together as folding proceeds. Some of them may not be bound by virtue of steric effect. By looking at the distribution of pairs of hydrophobic residues marked (×) in Fig. 9, we can see that the parts of (α_1–α_2), (α_3–α_4) and (β_1, β_2–α_5) form the domains respectively, because the parts between α_2 and α_3, and between α_4 and β_1 are rather long yet lacking enough hydrophobic pairs and consequently the folding of these parts take a rather long time, just as between the β-sheet and (α_3–α_4) in the case of lysozyme (see Section 5). Consequently we first make the bent structure of α_3 and α_4 which is obtained by changing the dihedral angles of the 58th residue and then fold the parts β_1, β_2, and α_5. The folded structure is shown in Fig. 10, where one can see that Cys 84 and 96 are sufficiently close to form the disulfide bond S_1. The selection of correct pairs of cysteines among possible ones must be made through more detailed examination of the geometrical relations of two cysteines, as will be discussed in III [2]. We then introduce the energy of the type eq. (1) between the side chains of Cys 84 and 96 replaced by the appropriate spheres. Next consider the contact between (α_3–α_4) and (β_1–β_2–α_5). We successively introduce S–S bonding energy to form S_2, S_3 and S_4 together with the hydrophobic and nonbonded energies between relevant atoms or groups. The conformation obtained by searching for the minimum energy is given in Fig. 11, where the folding between α_1 and α_2 is also undertaken,

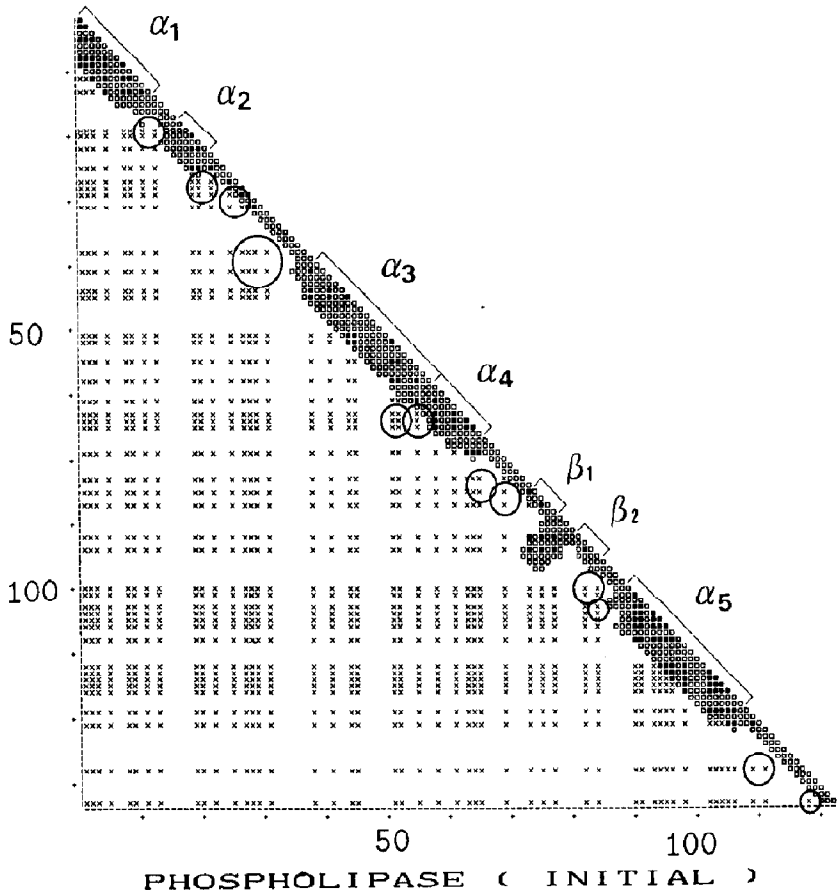


Fig. 9. Starting structure of phospholipase.

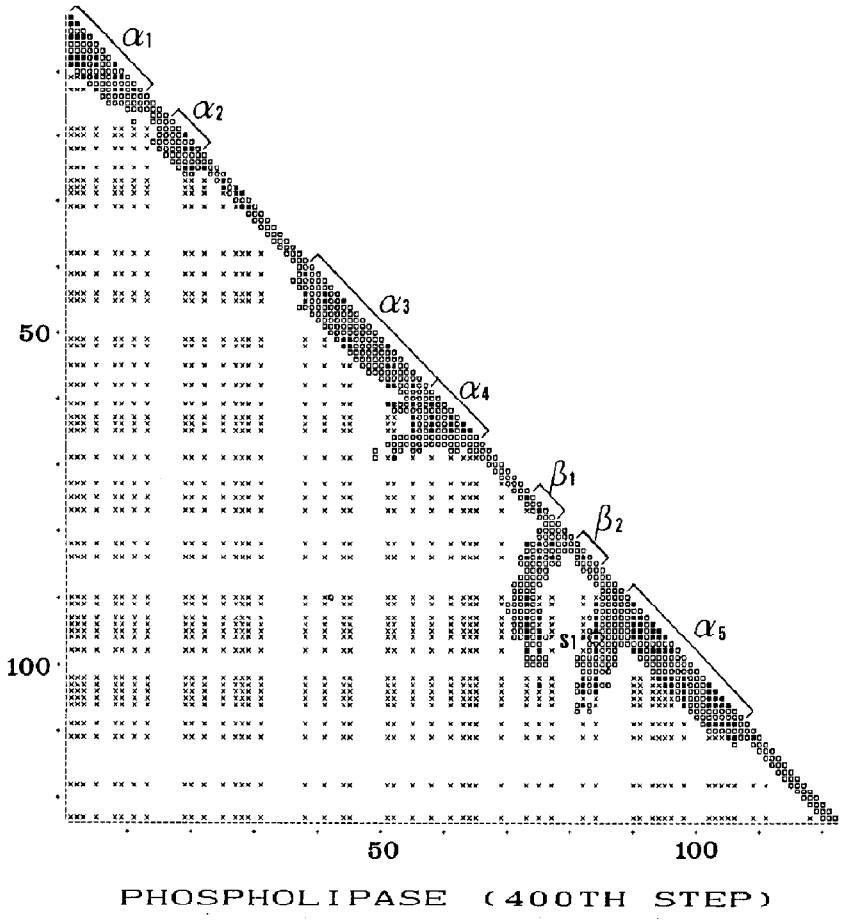


Fig. 10. Partially refolded structure of phospholipase (400th step).

which can be done independently of the conformation from α_3 to α_5. The last step is to search for the contact between two domains α_1–α_2 and α_3–α_5. This is performed by changing the dihedral angles of the part between α_2 and α_3. The disulfide bonds S_6 and S_7 are also taken into account. The final result is shown in Figs. 12 and 13. At the final stage (2000th step) the S_5 pair is not yet completed. This situation is similar to the final stage of folding in lysozyme.

4. Formation of antiparallel β-structures

A statistical mechanical method of predicting α-helices and β-strands is now being in progress by further development of the method of Wako et al. [7], and a new formulation by Saitô [8], but at the present stage the precision of prediction is not sufficient to be applicable to real proteins whose primary structures only are known. This situation is almost the same with other non-physical, statistical inference methods [9]. However, we are also required to elucidate the mechanism how a β-sheet can be constructed from neighboring β-strands once when they are determined. As concrete examples, we consider the cases in lysozyme and phospholipase. Lysozyme has five β-strands as mentioned in Section 1. The β_1 strand, which is rather separated from other β-strands, makes contact with the α_1-helix. We have only to consider β_2 through β_5. They are shown in Fig. 14(a). Phospholipase has two β-strands lying side by side, as shown in Fig. 14(b). It may be easily assumed that the hydrophobic interaction plays a role, as in the folding of secondary structures (α-helices and β-

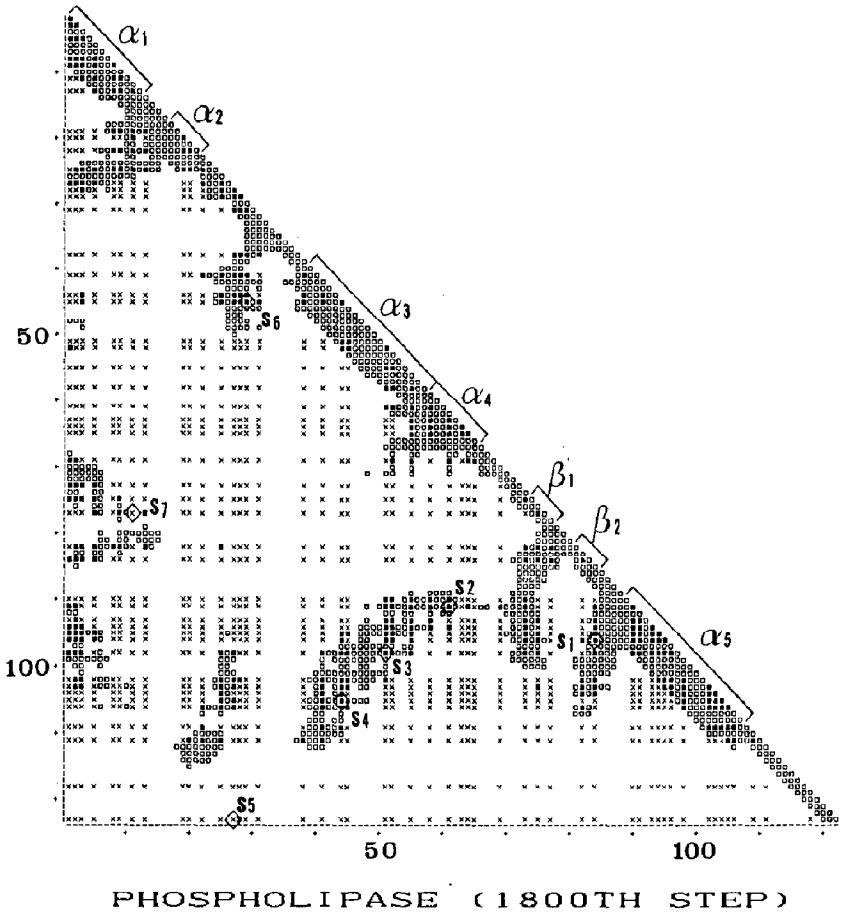


Fig. 11. Partially refolded structure of phospholipase (1800th step).

sheets) into tertiary structures discussed hitherto. In the native structure of lysozyme, antiparallel β-strands are formed between β_3 and β_4, and between β_4 and β_5 as shown in Fig. 14(a). One immediately sees that the Phe 38 and Ile 58 are the only hydrophobic residues in β-strands. Generally speaking, when hydrophobic residues are scarce, we may include Tyr, Cys and Ala in the group of hydrophobic residues, because Tyr has a side chain similar to Phe but with polar OH and Cys with polar SH of small dipole moment similar to Ala. The hydrophobic parts of these residues can give rise to weak hydrophobic interaction in agreement with the hydrophobicity values in Table 1 in I. This is also the case in erabutoxin for folding the tertiary structure, which will be discussed later in this series of papers. In this case the strengths of hydrophobic interactions between pairs with weak hydrophobic residues must be modified. Now we can understand the formation of the antiparallel β-structure between β_4 and β_5 in lysozyme by virtue of the interaction between Tyr 53 and Ile 58, as illustrated in Fig. 14(a). The same holds for phospholipase. The pairs of Cys 77 and Ile 82 and of Tyr 75 and Cys 84 can be bound by their hydrophobic interaction, as shown in Fig. 14(b). In these cases the hydrophobic residues are located on β-strands at the parts close to β turns, and when they approach, zipping of hydrogen bonding can take place. It is also to be noted that the β-turns are class 2 β-hairpins in phospholipase and class 4 β-hairpin in lysozyme in the terminology of Milner-White and Poet [10]. This difference may be attributed to the presence of or absence of

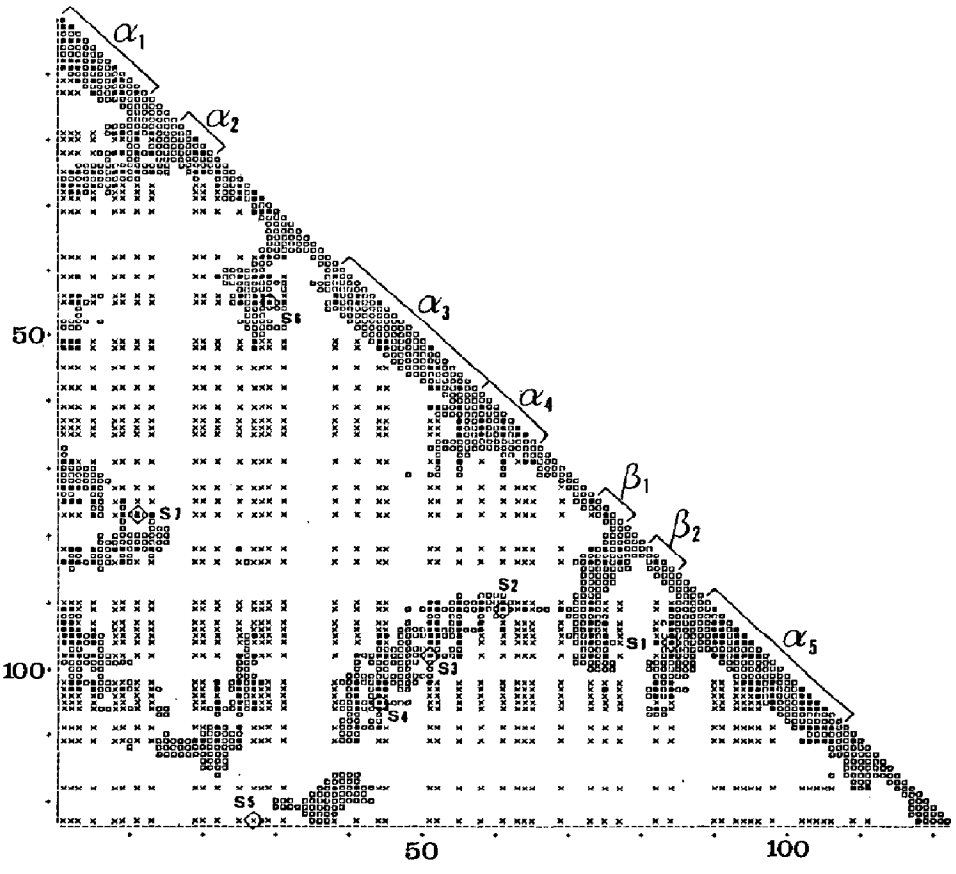


Fig. 12. Refolded structure of phospholipase (2000th step).

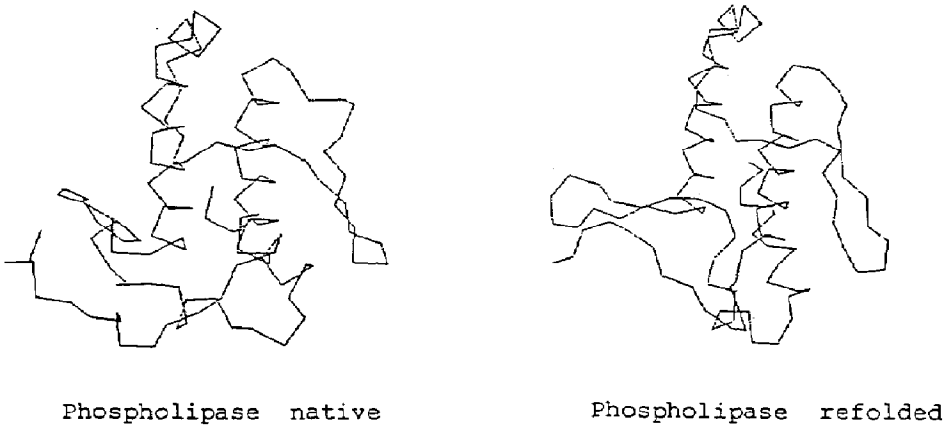
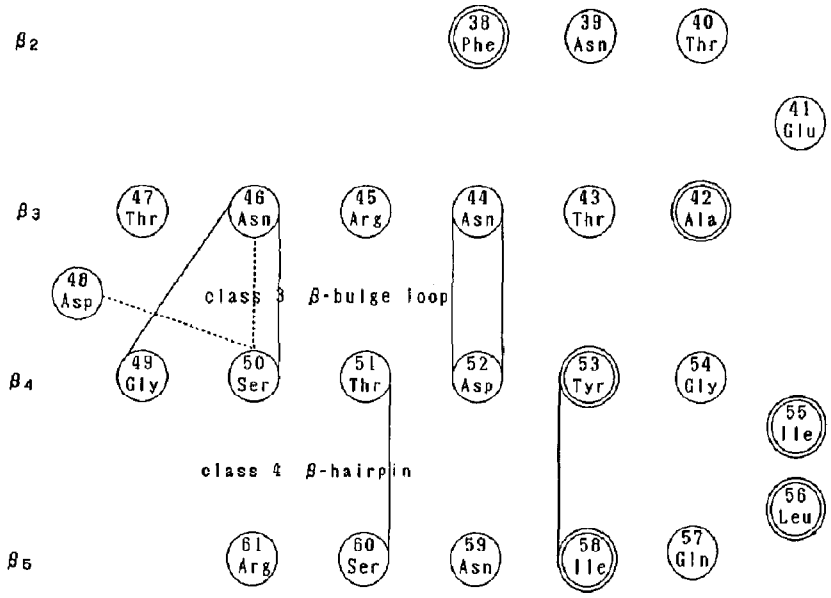
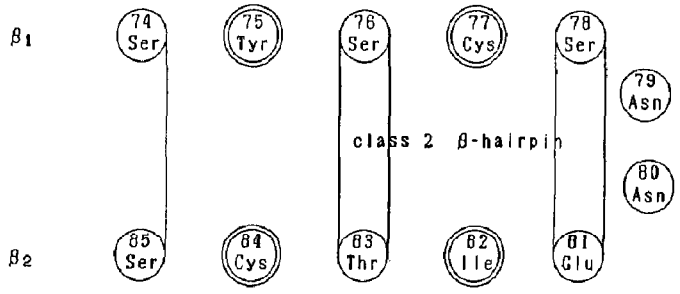


Fig. 13. Backbone structure of phospholipase for native (Fig. 8) and refolded (Fig. 12) structures.



(a) LYSOZYME (HEN EGG WHITE) 6LYZ



(b) PHOSPHOLIPASE 1BP2

Fig. 14. β-Structures in lysozyme (a) and phospholipase (b). (—) Hydrogen bond between main chain atoms, (· · ·) hydrogen bond between side chain, and (○) hydrophobic residues.

a hydrophobic interactions at the turn. In lysozyme two hydrophobic residues (Ile 55 and Leu 56) are situated side by side at the turn. The antiparallel β-structure between β_3 and β_4 in lysozyme, however, cannot be explained by the hydrophobic interaction. The β turn between β_3 and β_4 is a class 3β-bulge loop [10] (see Fig. 14a) with a hydrogen bond between CO as Asn 46 and N of Gly 49. There is also a hydrogen bond between N=O of the side chain of Asn 48 and O of the side chain of Ser 50 across Gly 49. The two hydrophobic residues Ala 42 and Tyr 53 are on the opposite side of the β-sheet. These two residues at the far ends of β-strands are not easily bound to yield an antiparallel β-structure of class 2 or class 4 β-hairpin which might be expected to occur just as between β_4 and β_5. The β-bulge loop between β_3 and β_4 is formed by the two hydrogen

bonds mentioned above. No antiparallel β-structure can be expected between β_2 and β_3 because there seems no driving force for this structure. This is in fact the case as one can see from Fig. 1. The mechanism of the formation of an antiparallel β-sheet, however, is not yet completely elucidated. There exist some examples which do not fall into the categories mentioned above. More research is needed.

5. Discussion

The folding order is sometimes important to obtain the refolded structure. This is the case also in myoglobin. In the previous paper I that discussed myoglobin, no explicit mention was made of the folding order, but we had to first construct two domains composed of the A–E helices and the F–H helices, and then make contact between these two domains by changing the dihedral angles of the part between E and F. Otherwise the correct topological arrangement between A–B helices and E–F helices would not be obtained. We have to ask the reader to refer to Fig. 1 in I to understand that this procedure is legitimate. The part between C and D composed of 8 amino acid residues has plenty of hydrophobic residues and is easily and quickly folded, but the part between E and F composed of 8 amino acid residues have no pairs of hydrophobic residues. The part between G and H also lacks hydrophobic residues but the number of amino acid residues is five. Consequently the last folding takes place at the part between E and F. In the case of lysozyme the refolding of the part (61–80) between the β-sheet (composed of β_3, β_4, and β_5) and α_3 is not easy, compared with the refolding of the parts (16–24) and (85–89), by virtue of the scarcity of the hydrophobic residues (Trp 62, Trp 63, Leu 75 and Ile 78 only) and rather long distance (20 residues) as can be seen in Figs. 1 and 2. This implies that the two parts from α_1 to β_5 and from α_3 or α_4 are formed first and then they are combined. The α_5 and α_6 helices make contact with α_1 and α_2 in the final stage. This also indicates the sequence of disulfide formation: S_1, S_2, S_3 and finally S_4. This is in agreement with the experimental study on disulfide formation by Anderson and Wetlaufer [11]. In the previous computer simulations of unfolding of myoglobin and lysozyme the initial unfolding is shown to take place at the part between the E and F helices in myoglobin and at around 100–102 residues in lysozyme [12]. These facts are in agreement with the above considerations.

In this connection, mention must be made of the extensive experimental studies on lysozyme by Acharya and Taniuchi [13]. In particular, we notice two facts, (i) non-random formation of disulfide bonds and (ii) non-existence of an intermediate state with one open disulfide bond between Cys 30 and Cys 115(S_3). The non-randomness of disulfide bonding is the subject of our main concern. We have shown the sequence of formation of disulfide bonds. However, this sequence is not necessarily very strict in some cases, as will be briefly discussed below for phospholipase.

The intermediate state with the open S_3 bond cannot exist because this bonding is necessary for the S_4 bond as discussed above, but either S_1 or S_2 is sufficient for S_3 bonding, because as can be seen in Fig. 3, only hydrophobic interactions (and non-bonding interactions) bring the S_1 and S_2 pairs close enough to be bonded and consequently S_3 and S_4 bonds will result by either a S_1 or S_2 bond.

In the case of phospholipase, the formation of disulfide bonds will be done in two series of reactions, one is S_1, S_2, S_3, S_4 and the other is S_6, S_7 and S_5. In an actual process, the formation of S_6 will be inserted somewhere in the first series, because the packing of α_3, α_4 and α_5 can be made independently of that of the part $\alpha_3 \sim \alpha_5$. As far as the present authors are aware, no experimental study on the refolding of phospholipase has been published.

In the folding process we have assumed the rapid formation of secondary structures prior to formation of the tertiary structure. This fact has been experimentally verified by many authors for various proteins, especially for lysozyme by Kuwajima et al. [14]. It is now established that the A state (termed by Kuwajima et al. [14,15]) or molten-globule state (termed by Ohgushi and Wada [16] and Dolgikh et al. [17]) exists as an

intermediate state similar to native secondary structures without tertiary structure, see also Mitaku et al. [18].

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