Circular Permutation Analysis as a Method for Distinction of Functional Elements in the M20 Loop of Escherichia coli Dihydrofolate Reductase

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A functional element of an enzyme can be defined as the smallest unit of the local peptide backbone of which the connectivity is crucial for the catalytic activity. In order to elucidate the distribution of functional elements in an active site flexible loop (the M20 loop) of Escherichia coli dihydrofolate reductase, systematic cleavage of main chain connectivity was performed using circular permutation. Our analysis is based on the assumption that a permutation within a functional element would significantly reduce enzyme function, whereas ones outside or at the boundaries of the elements would affect the function only slightly. Thus, a functional element would be assigned as the minimum peptide chain between the identified boundaries. Comparison of the activities of the circularly permuted variants revealed that the peptide chain around the M20 loop could be divided into four regions (regions 1–4). Region 1 was found to play an important role in overall tertiary fold because most variants permuted at region 1 did not accumulate in E. coli cells stably. A distinction between region 2 and region 3 was in agreement with the extent of movements calculated from the coordinates of α carbons, supporting the idea that the movement of peptide backbone is a key feature of enzyme function. The boundary between region 3 and region 4 coincided with that between the M20 loop and the following α helix. From equilibrium binding studies, region 2 was found to be involved in the binding of nicotinamide substrates, whereas region 4 appeared to be very important for the binding of pterin substrates.

The active site of an enzyme contains amino acid residues involved in enzyme function. Some residues in the active site bind to the substrate or cofactor, and others are involved in the catalysis itself. In addition, residues away from the active site sometimes promote the catalytic reaction through intramolecular interactions (1, 2). Enzyme-catalyzed reactions proceed, in general, through multiple steps, including binding of the substrate, catalysis, and release of product. At each step, certain amino acid residues play critical roles. However, an isolated collection of these directly functioning residues is not sufficient to obtain catalytic activity. Amino acid residues should be connected covalently to make up a polypeptide chain with a specific amino acid sequence that determines a proper tertiary structure. The functioning residues on the peptide backbone are then arranged to give the proper configuration for effective catalytic activity. Because enzymes cleaved at certain sites have been demonstrated to show catalytic activity as high as that of uncleaved enzyme (3–16), chain connectivity must not be absolutely required for catalytic activity. This means that chain connectivity is crucial for catalytic activity in some regions, but in others, it is not. The detailed configuration of the functioning residues seems to depend on local peptide backbone rather than the overall polypeptide chain. In this paper, we define a “functional element” as the smallest unit of the local peptide backbone of which the connectivity is crucial for the catalytic activity. Such functional elements would be distributed across the primary structure. When the enzyme is properly folded, these elements would associate to make a functional active site (Fig. 1). Identifying such functional elements would be very informative in revealing the architecture of enzyme function. Here, we propose a novel approach for identifying the functional elements of an enzyme.

Based on our definition of a functional element, alteration of main chain connectivity (peptide bond cleavage) seems to be a useful approach for locating functional elements. Possible consequences of peptide bond cleavage are illustrated in Fig. 1. If the main chain is cleaved within a region that is indispensable for protein folding (designated as the folding element), the resulting protein will not be able to fold to a stable conformation (case 1). In the other cases, the properties of the cleaved protein would vary depending on the location of the cleavage site. Cleavage within a functional element (case 2) will break the local configuration, leading to loss of the function contributed by the element, whereas those outside (case 3) or at the boundary of (case 4) the functional elements will not, resulting in a minor effect on the enzyme function. Even if functional elements are located side by side, distribution of the functional elements can be recognized by the presence of the boundaries (compare case 2 and case 4). Based on this idea, systematic peptide bond cleavage and characterization of the products are crucial to locating functional elements. The simplest way to cleave a peptide bond is to break the polypeptide chain into two fragments by manipulation of the coding gene. However, this dissection method is poorly suited for the purpose of obtaining a variety of cleaved proteins without disrupting the overall tertiary fold, because production of properly folded protein requires the association of the fragmentary chains by long-range interaction. Circular permutation analysis, in which the original N and C termini of a protein are connected by an appropriate linker and new termini are created at a position of interest (Fig. 1), can overcome such entropic problems because it allows a peptide bond to be broken without fragmenting the protein into two pieces. To date, a number of circularly per-
mutations of proteins with various folded structures have been reported (3–16). Construction of circularly permuted (CP) variants has revealed that the N and C termini of proteins can be moved to alternative positions without lethal damage and that the order of peptide synthesis is not critical for the final tertiary structure of a protein. In most cases, newly created termini were directed to sites such as interdomain hinges or surface loops. On the other hand, random circular permutation was applied to the catalytic chains of aspartate transcarbamoylase to investigate possible rules for inserting termini in various regions of the three-dimensional structures of proteins (8). In the same way that alanine scanning has been used to elucidate the role of side chains (17), systematic circular permutation analysis will be useful for estimating the role of main chain connectivity.

We chose an active site flexible loop of Escherichia coli dihydrofolate reductase (DHFR) (EC 1.5.1.3) as the target of an investigation of functional elements. DHFR catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) and plays an important role in supplying the cofactor for one-carbon transfer reactions, e.g. the reaction catalyzed by thymidylate synthase (18). The flexible loop connecting β strand A and α helix B of E. coli DHFR (Ala-9-Leu-24) has been called loop I (19) or the M20 loop (20) (Fig. 2). This loop has attracted much attention because of its variable con-

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**Fig. 1.** Schematic drawing of functional elements and strategy of circular permutation. Shaded lines indicate functional elements, boldface lines are the peptide linker used in circular permutation, and scissors show the positions of main chain cleavage. Cleavage within a region that is important for the tertiary fold breaks the overall structure (case 1). Cleavage within a functional element results in a less active variant (case 2). Cleavage outside (case 3) or between (case 4) functional elements affects the enzyme function only slightly.

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**Fig. 2.** Drawing of the tertiary structure of DHFR using Protein Data Base file 1rx1. Four α-helices (αA, αC, αE, and αF) and eight α-structures (βA, βB, βC, βD, βE, βF, βG, and βH) are shown by ribbons. The peptide region from Leu-8 to Asp-27 is indicated by the black area. Positions of Leu-8 and Asp-27 and their side chains are shown by arrows and by balls and sticks, respectively. The positions of the N and C termini are also indicated.

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**EXPERIMENTAL PROCEDURES**

**Materials**—E. coli JM109 was used as cloning and expression host. Methotrexate affinity resin was obtained from Sigma. DEAE-Toyopearl 650 M was purchased from Tosoh Co. (Tokyo, Japan). Restriction enzymes, T4 DNA ligase, and Taq polymerase were obtained from Takara Shuzo Co. (Kyoto, Japan). All primer DNAs for mutagenesis were synthesized by JbioS Ltd. (Saitama, Japan). All other chemicals were of reagent grade.

Construction and Purification of Circularly Permutated Variants—
Construction of the circularly permuted genes was carried out as described previously (6). Coding sequences of circularly permuted proteins were amplified with polymerase chain reaction on a tethered dimer gene. Then, an overexpression promoter, a ribosome-binding site, and BamHI sites were attached to the coding sequence. Resulting genes were thereafter cloned into BamHI site of a high copy vector, pUC19, plasmid, sequenced with the dideoxy method to confirm the construction. E. coli JM109 cells were transformed with the expression plasmid and tri-methoprim (TMP) resistance was tested on the agar plate as described previously (6). CP variants giving TMP resistance were further purified. Purification of the CP variants was carried out mainly by methotrexate affinity chromatography, taking advantage of adequate purification steps from cell-free extracts (26). Protein concentration was determined by the absorbance at 280 nm using the extinction coefficient ($\epsilon_{280} = 31,100 \text{ M}^{-1} \text{cm}^{-1}$ (27)) for wild-type and circularly permuted DHFR.

**Enzyme Assay**—The activity of DHFR was determined spectrophotometrically at 15 °C by following the disappearance of NADPH and DHF at 340 nm ($\epsilon_{340} = 11,800 \text{ M}^{-1} \text{cm}^{-1}$ (28)). The standard assay mixture contained 50 μM DHF, 100 μM NADPH, 14 mM β-mercaptoethanol, MTEN buffer (50 mM 2-mercaptoethanol, 3 mM EDTA, 25 mM tri-hydroxymethylaminomethane, 25 mM ethanolamine, and 100 mM NaCl, pH 7.0 (29)), and the enzyme in a final volume of 2.0 ml. The reaction was started by the addition of DHFR.

**Circular dichroism**

Circular dichroism spectra were obtained by scanning from 250 to 190 nm with 15 or 20 s averaging times on an Aviv 62DS spectrometer using either a 1- or a 2-mm cuvette. Protein concentrations were 0.05–0.2 mg/ml. The raw data were converted to mean residue ellipticity (MRE) by the equation $\text{MRE} = \Theta (\theta = 100(C \times D \times \text{NA})$, where $\Theta$ is the ellipticity in degrees, $C$ is the molar protein concentration, $D$ is the path length in cm, and NA is the number of residues in the protein.

**Urea Denaturation**—Urea-induced unfolding of proteins was studied by monitoring ellipticity at 222 nm. All samples were dialyzed against 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM β-mercaptoethanol. The proteins were diluted to varying final urea concentrations in the above buffer and incubated at least 12 h prior to the data collection. Final protein concentrations ranged from 0.1 to 0.5 mg/ml. Equilibrium unfolding data obtained from the ellipticity at 222 nm were analyzed based on a two-state model as described previously (30, 31).

**Extent of the Movement of α-Carbons**—Coordinates of α carbons were obtained from Protein Data Base codes 1rx1, 1rx4, and 1rb3. Conformations of the M20 loop in these structures are closed, occluded, and open, respectively. Calculations were carried out using the AMBER program (Oxford Molecular).

**Alanine Scanning Experiments**—Mutant genes with single alanine substitutions in the region from Leu-8 to Asp-27 (excluding Ala-9, Ala-19, and Ala-26), were constructed and expressed in E. coli cells (alanine scanning) according to a similar method described previously (32). For Ala-9, Ala-19, and Ala-26 sites, glycine scanning was performed. All the mutant proteins were overexpressed and were found to accumulate stably in E. coli cells. The relative activity of the mutant proteins were estimated as follows: each E. coli transformant was grown in 2 ml of 2YT culture medium (10 g/l NaCl, 16 g/l tryptone, 10 g/l yeast extract) containing 200 mg/ml of ampicillin, at 37 °C, until the absorbance at 600 nm reached approximately 1.5. One ml of cell culture was harvested by centrifugation, and the cells were suspended in 0.2 ml of 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA containing 1 mg/ml lysozyme. After gently mixing for 30 min, cells were disrupted by sonication for 10 s. Cell debris was removed by centrifugation, and 10 μl of the resultant supernatant was used for the DHFR assay. To normalize the activity to the number of cells used, the measured activity ($\Delta A_{340}/\text{min}$) was divided by the absorbance at 600 nm ($\Delta A_{600}/\text{min}$). Enzyme activity relative to wild-type DHFR is defined as the ratio of $\Delta A_{340}/\text{min}$/$\Delta A_{600}$ for the mutant protein to $\Delta A_{340}/\text{min}$/$\Delta A_{600}$ for the wild-type DHFR.

**Others**—Equilibrium dissociation constants ($K_d$) were determined from fluorescence emission spectra of enzyme-ligand complexes using an Aviv ATF105 spectrophotometer. The solution contained 1 μM enzyme and various concentrations of ligand (DHF, THF, NADPH, or NADP+) in MTEN buffer, pH 7.0. The emission spectra were obtained from 500 to 800 nm at 15 °C, with an excitation wavelength of 290 nm. DNA sequencing was carried out using an ABI PRIZM 310 genetic analyzer. N-terminal amino acid sequence determination (Edman degradation) and mass spectrometric measurements were carried out as described previously (6). NADPD was prepared using alcohol dehydrogenase from Thermoanaerobrio brockii (Sigma) and purified on a Mono-Q column (Amersham Pharmacia Biotech).

**RESULTS**

**Production of Circularly Permuted Variants**—All circularly permuted genes were created by polymerase chain reaction on a tethered dimer gene. Because a five-glycine peptide had been shown to be the most favorable linker in the circularly permuted DHFR with N terminus at Met-16 (cpM16; similar abbreviations are used for all CP variants unless noted) (6), this peptide linker was employed in all variants (from cpL8 to cpD27). For the construction of the circularly permuted genes (except that of cpM16), the codon for methionine (ATG) was introduced at the 5′-ends of the coding sequences for translation initiation. For cpM16, because the codon for Met-16 was also used as the initiation codon; an extra ATG initiation codon was not added. However, an extra ATG codon was needed for the gene of cpM20, because the N terminus of the purified cpM20 was Pro-21 that was resulted from the removal of Met-20 by the methionyl-aminopeptidase of E. coli (33). The constructed genes were cloned into a high copy plasmid, pUC19, sequenced, and used to transform E. coli JM109. The E. coli transformants harboring DHFR activity were selected for on agar plates containing TMP, a competitive inhibitor of DHFR. The transformants corresponding to from cpL8 to cp14 were TMP-sensitive, whereas those corresponding to from cpG15 to cpD27 were TMP-resistant. The mutant proteins at the positions from Leu-8 to Ile-14 could not be obtained because of poor accumulation in the E. coli cells as observed by SDS-polyacrylamide gel electrophoresis of the crude extracts. This suggests that peptide connectivity of the region is crucial for protein folding, namely, it is a folding element. Based on these results, the peptide chain around the M20 loop could be classified into two regions separated by a boundary at Ile-14 or Gly-15. Then, permutant proteins from cpG15 to cpD27 were purified and further analyzed. N-terminal amino acid sequences and molecular masses were in agreement with the sequences of the constructed genes (Table I). According to re-action of methionyl-aminopeptidase (33), the N-terminal methionines of the translated polypeptides may or may not have been processed. Specifically, N-terminal methionines were observed in cpM16, cpE17, cpN18, cpM20, cpW22, cpN23, cpL24, and cpD27 but not in cpG15, cpA19, cpP21, cpP25, or cpA26.

In our study, the purpose of the circular permutation is to investigate whether or not the cleavage of chain connectivity at each position breaks a functional element. Therefore, the position of the cleavage is an essential consideration in the construction of variants, but the identity of the N-terminal residue is not. Nevertheless, in order to assess the effect of the extra N-terminal residue, we constructed and characterized variants in which Met and Ala were added to the N terminus of cpM16 and cpP21, respectively. Thus, these new variants were designated as cpMM16 and cpMAP21, respectively. Measurements of the activities of cpMM16 and cpMAP21 showed that the N-terminal structure was not critical for the properties of CP variants (see below).

**Enzymatic Activity of Circularly Permuted Variants**—The activity profiles of CP variants are shown in Fig. 3. When the $k_{cat}$ values were plotted against the position of the new termini in the original loop, a significant landscape with three peaks was observed. Because peaks were observed at cpM16, cpM20, and cpL24, cleavages of peptide bonds at Met-16, Met-20, and Leu-24 did not have a significant effect on the catalytic function. If we let these three points define the boundaries (boundaries I, II, and III; see Fig. 3), then the peptide chain around the M20 loop could be divided into four regions. Boundary I, which was recognized from the minor peak at cpM16, is close to the N-terminal residue of the protein. Thus, the region N-terminal to boundary I seems to
involve several residues. Boundaries II and III, which were assigned based on the major peaks at cpM20 and cpL24, respectively, clearly divide the peptide chain into multiple functional elements. The \( k_{\text{cat}} \) value for cpMM16 and cpMAP21 were 0.4-fold and 3.0-fold of cpM16 and cpP21, respectively (data not shown). Replacement of cpM16 and cpP21 by cpMM16 and cpMAP21, respectively, did not change the characteristics of the plot significantly. Therefore, the boundaries determined from the plot of Fig. 3 are valid even though the terminal structures are not identical among the CP variants. As a result, we assigned the four regions as follows: region 1, Met-16; region 2, Met-16–Met-20; region 3, Met-20–Leu-24; and region 4, Leu-24–.

**CD Spectra**—Far UV CD spectra of CP variants are shown in Fig. 4. A mutant DHFR with a five-glycine peptide at its C-terminal end, cpM1, was used as a control (6). The spectra of the variants from cpM16 to cpN23 were quite similar to that of cpM1. This result supports the notion that peptide bond cleavage at the positions Met-16 through Asn-23, which are within the flexible loop, does not cause a significant difference in the overall backbone structure. However, the spectrum of cpG15 was somewhat different from that of the control and suggested a less structured form. This observation, together with poor accumulation of the variants from cpL8 to cpI14, suggests that region 1 plays an important role in allowing DHFR to fold to the correct tertiary structure. CD spectra of the variants from cpL24 to cpD27 showed different patterns from that of the control, suggesting a decrease in the \( \alpha \)-helical content. This is likely due to the perturbation of a helix B, which extends from Leu-24 to Asn-34.

**Urea-induced Unfolding**—The urea-induced unfolding of the CP variants showed cooperative two-state transitions like the wild-type. Thus, the folded structure of the variants could not be considered a “molten globule.” The thermodynamic parameters for the unfolding are listed in Table I. For most variants,
Mimicking enzyme catalysis is one of the ultimate goals of biochemical engineering. Among the many attempts to design an artificial enzyme, utilization of immune system has been the most effective strategy. However, acceleration of the reaction in terms of catalytic efficiency is less effective than the natural enzymes. Because the enzyme reaction can be divided into multiple steps, the function of the enzyme should also be a combination of multiple factors. Therefore, this study aimed to identify the functional elements and their roles in the reaction process.

### Role of the Functional Elements

In order to determine whether deuterium isotope effect measurements were made for the CP variants (Table I). In wild-type DHFR, the isotope effect was close to 1 because the rate-limiting step at pH 7.0 is the release of THF (25). In the CP variants, increased isotope effect values were observed in the less active variants. Thus, the decreased activity observed for the CP variants were due to the hydride transfer step. On the other hand, distinct roles in ligand binding were identified for each functional element. In cpN18, cpW22, and cpD27, regions 2, 3, and 4, respectively, are expected to be broken by the insertion of new termini. Equilibrium dissociation constant ($K_d$) values of the wild-type and these CP variants are listed in Table II. In cpN18, $K_d$ values for NADPH and NADP$^+$ were much higher than those in the wild-type, whereas those for DHF and THF were quite similar to the wild-type. In cpW22, although $K_d$ for NADPH was 10-fold higher than the wild-type, the differences were moderate when compared with the other variants. In cpD27, $K_d$ values for DHF and THF were extremely high, whereas those for NADPH and NADP$^+$ were not dramatically different. These results suggest that region 2 is responsible for the binding of nicotinamide substrates and that region 4 is responsible for binding of pterin substrates.

### Alanine Scanning Experiment

In contrast to circular permutation, amino acid replacement does not alter the chain connectivity. The effects of single amino acid replacements with alanine (or glycine for Ala-9, Ala-19, and Ala-26) in the region from Leu-8 to Asp-27 were examined. Fig. 6 shows the relative enzyme activity of the mutant proteins. In this experiment, cell free extracts were used to perform the enzyme assay, because, unlike the circular permutants, all of the singly substituted proteins accumulated at high levels in E. coli cells. Furthermore, the expression levels were similar to each other, as determined by SDS-polyacrylamide gel electrophoresis of the crude extracts. The activity of I14A, E17A, W22A, and D27A mutants was greatly decreased by the mutation, whereas that of V10A, M16A, A19G, N23A, L24A, and P25A mutants was higher than that of the wild-type enzyme. The boundaries, I, II, and III, which were suggested by the circular permutation analysis, are also suggested by this alanine scanning experiment, although they are less obvious than in the circular permutation analysis.

### Discussion

Mimicking enzyme catalysis is one of the ultimate goals of biochemical engineering. Among the many attempts to design an artificial enzyme, utilization of immune system has been the most effective strategy. However, acceleration of the reaction in terms of catalytic efficiency is less effective than the natural enzymes. Because the enzyme reaction can be divided into multiple steps, the function of the enzyme should also be...
boundaries I, II, and III, suggested in Fig. 3, respectively.

Leu-8 to Asp-27.

and/or stable variant. In our study, the variants from cpL8 to peptide bonds in a protein will not always generate an active permuted proteins have been reported, cleavage of one of the circular permutation analysis for the discrimination of functional elements.

manipulation of main chain connectivity, but not of side chains, would be helpful. In this paper, we described the utility of circular permutation analysis for the discrimination of functional elements.

Although successful constructions of a number of circularly permuted proteins have been reported, cleavage of one of the peptide bonds in a protein will not always generate an active and/or stable variant. In our study, the variants from cpL8 to cpI14 could not be obtained because the corresponding E. coli transformants were TMP-sensitive. This result suggests that the chain connectivity of this region is necessary for the formation of stable tertiary structure, namely, folding of the enzyme (Fig. 1, case 1). On the other hand, peptide bond cleavage at each position between Gly-15 and Asp-27 yielded active and stable variants, cpG15–cpD27. Successful construction of these variants enabled the systematic analysis of the chain connectivity at the M20 loop.

Comparison of the activities of CP variants (Fig. 2) revealed three boundaries that divide the peptide chain around the M20 loop into four regions (region 1, Met-16; region 2, Met-16–Met-20; region 3, Met-20–Leu-24; region 4, Leu-24–). These boundaries were also suggested, but in a more subtle fashion, by the alanine (and glycine) scanning experiments (Fig. 6). The variants permuted at region 1 were not studied, except for cpG15, because of poor accumulation in the transformed E. coli cells. For cpG15, the far UV CD spectrum suggested that the main chain fold was not similar to the wild-type (Fig. 3), although Gly-15 is located in the middle of the M20 loop. Thus, region 1 was characterized as a region important for tertiary structure formation of the enzyme (i.e., a folding element) rather than as a functional element. The other regions, regions 2, 3, and 4, were clearly assigned as functional elements around the M20 loop. Although deuterium isotope effect measurements did not identify the catalytic step contributed by the functional elements, studies on the equilibrium dissociation constants suggested that these regions play distinct roles in ligand binding (Table II). Specifically, region 2 was shown to be involved in binding of NADPH and NADP⁺, whereas region 4 was involved in binding of DHF and THF. These tendencies were in agreement with the crystallographic data available from Protein Data Base files 1rx1, 1rx9, 1rx7, and 1rx5, which represent wild-type DHFR complexed with NADPH, NADP⁺, folate, and 5,10-dideazatetrahydrofolate, respectively. Although the characteristics of the regions in ligand binding were similar to those obtained by crystallographic data, experimental materials in which chain connectivity at various positions are systematically perturbed are provided solely by the use of circular permutation. Therefore, circular permutation analysis gives an experimental approach to elucidate the role of functional element.

Detailed characteristics of the conformation around the M20 loop also supported the division of the loop into multiple elements. The conformation of the M20 loop in the crystals has been classified into three types: closed, occluded, and open conformations (22). In the closed conformation, Met-16–Ala-19 forms an antiparallel sheet and type III’ hairpin. In the occluded conformation, Glu-17–Met-20 form a 3₁₀ helix. In the open conformation, although the conformation is irregular, the side chains of Met-16 and Met-20 form stabilizing hydrophobic contacts within the loop. The above regions all coincided with region 2. From the coordinates of α carbons forming the M20 loop in these conformations, the extent of the movements of each residue could be compared (Fig. 5). If the M20 loop moves as a single string that cannot be further divided, the shape of the plot of movement versus residue number should consist of single peak. However, the shape of the plot consisted of two peaks around Glu-17 and Pro-21 (Fig. 5). This means that regions of the peptide chain around Glu-17 and Pro-21 move separately. These segments, which were distinct in terms of movement, correspond to two functional elements, region 2 and region 3. The finding that the unit of movement and the unit of functioning peptide (functional element) coincided supports the idea that movement of the peptide backbone is a key feature of enzyme function.

There have been many attempts to understand a protein as being composed of multiple segments. From the tertiary structure of a protein, domains and secondary structural units can be easily recognized. Additionally, compact structural units called “modules” are found by an algorithm using the coordinates of Cα atoms (35, 36). In addition to the computational methods, an experimental method to identify the segments of the protein is the modification on the main chain connectivity. The simplest way to modify main chain connectivity is to dissect the polypeptide at a certain position by fragmentation of the coding gene. When the dissection occurs at the boundary of proper segments such as domains (37, 38), modules (39), and exon-coded segments (40), the peptide fragments may associate to give an active complex molecule as high as the activity of the parental protein. Circular permutation is a useful alternative to cleaving main chain connectivity because it does not cause fragmentation of the polypeptide chain. Using this method, we detected multiple functional elements in the M20 loop of E. coli DHFR. Similarly, by examining whether the CP variant can fold stably, peptide regions that play important roles in stable folding (folding elements) can be distinguished from the other regions that do not. Peptide bond cleavage within folding elements will prevent the folding to stable tertiary structure, whereas cleavage outside the folding elements will not. Based on this idea, the M20 loop could be divided not only into functional elements but also into folding elements. Among the regions divided in this study, region 1 can be assigned as a folding element, whereas, region 4, which does not belong to a folding element, forms a helix B. This observation suggests that folding elements are not always characterized as a unit of secondary structure. Systematic construction of the CP variants at all the positions of DHFR (namely, 158 sites) has been completed and provided an overview of folding elements in the enzyme (41). Because circular permutation has been reported for many proteins other than DHFR (3, 7–16), the approach used in this study should be generally applicable to many proteins.

Acknowledgments—We thank Mr. Hisashi Takahashi, Nagaoka University of Technology, for the calculations of the movements of α car-

![Fig. 6. Alanine and glycine scanning plot at the region from Leu-8 to Asp-27. Arrows marked I, II, and III indicate the positions of boundaries I, II, and III, suggested in Fig. 3, respectively.](image-url)
bons and Tatsuyuki Takenawa, National Institute of Bioscience and Human-Technology, for mass spectrometric measurements. We are grateful to Prof. C. Robert Matthews and Dr. Virginia F. Smith, the Pennsylvania State University, for continuous discussion and encouragement and for critical review of this article.

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