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Stability and Reversibility of Thermal Denaturation Are Greatly Improved by Limiting Terminal Flexibility of *Escherichia coli* Dihydrofolate Reductase

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Short peptides which contained a single Cys residue were introduced into both N- and C-termini of the Cys-free mutant of DHFR (Cys85→Ala, Cys152→Ser double mutant) by a recombinant DNA method, then the terminal regions were connected through a disulfide bond by oxidation. The oxidized form and reduced form proteins have as high enzymatic activity as wild-type DHFR. There is no detectable difference between the CD spectra of the reduced and oxidized forms at low (15°C, native condition) and high temperature (80°C, unfolded condition). The thermal transition of the oxidized proteins at the concentration of 0.15 mg/ml (8.5 μM) is completely reversible as demonstrated by the CD spectra. No aggregated materials were detected in the oxidized protein on gel-filtration HPLC after heat treatment up to the protein concentration of 0.5 mg/ml. The reduced protein, however, even in the presence of reducing agent, showed only partial reversibility, with as much as 55 and 95% of the heat-treated protein at the concentrations of 0.15 and 0.5 mg/ml being eluted as the high molecular aggregated form, respectively. The apparent transition temperatures (T_m) of the oxidized forms were 5-7°C higher than those of the reduced counterparts. The oxidized protein that had been denatured with guanidine-HCl was eluted later than the denatured reduced protein on gel-filtration HPLC in the presence of 5 M guanidine-HCl. The limitation of spatial movement of the termini may prevent intermolecular interaction of exposed domains during denaturation-renaturation process, giving rise to the irreversible denaturation. The flexibility of the terminal is also suggested to be an important factor for improving thermal stability of proteins.

Key words: circularized protein, dihydrofolate reductase, *Escherichia coli*, reversibility, thermal stability.

Thermal denaturation of many proteins often causes irreversible aggregation and inactivation (*1*). This makes it difficult for reversible control of an enzymatic reaction by physical means such as temperature shift, even though such on-off regulation is attractive in practical use of protein catalysts. In contrast to the improvement of thermal stability by protein engineering, few works have aimed to improve thermal reversibility (*2*), nor has any general approach been successfully made.

The terminal regions of proteins are generally more flexible than other parts. However, there is a significant preference for terminal regions in single-domain proteins to be in close proximity (*3*). An increase in flexibility in terminal regions may enhance intermolecular interactions of exposed domains during the denaturation-renaturation process. If so, the reversibility must be improved by reducing the flexibility at the terminal regions, *e.g.*, by covalently connecting N- and C-termini through an extra loop.

To test this approach, dihydrofolate reductase (DHFR, EC 1.5.1.3) has been engineered so that the N- and C-termini are connected with a peptide chain containing a disulfide bond. DHFR catalyzes the 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 (*4*). Its crucial role in metabolism, relatively small size, 18 kDa, and monomeric structure have made DHFR the subject of a variety of biochemical, biophysical, and protein engineering studies. DHFR from *Escherichia coli* is denatured by temperatures higher than 40°C. This denaturation is only partially reversible and is accompanied by aggregation and precipitation (*5*). An engineered disulfide bond inside DHFR, which was designed to reduce local flexibility, does not improve the thermal stability nor the reversibility, although overall conformational stability as measured by reversible guanidine hydrochloride denaturation is apparently improved (*5*). To reduce the flexibility of the terminal regions, the direct connection of the N- and C-terminal amino acids by a peptide bond is the simplest way, as has been successfully carried out in BPTI (*6*). However, this method is not applicable to DHFR, because the α-carbons of its N- and C-terminal amino acids are separated by as much as 15 Å, similar to the radius of gyration [*ca.* 17 Å of the wild-type-DHFR (Iwakura *et al.*,

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unpublished observation)]. As an alternative method, we introduced short peptides containing a single Cys residue into both N- and C-termini of the DHFR, then connected the terminal regions through a disulfide bond between the two Cys residues in the introduced peptides. This approach was successful, and it demonstrated that a limitation of spatial movement of terminal regions is crucial to the thermal reversibility of DHFR.

MATERIALS AND METHODS

Chemicals—Methotrexate-agarose affinity resin was obtained from Sigma. DEAE-Toyopearl 650M and Toyopearl HW55 from Tosoh (Tokyo). All other chemicals were of reagent grade. Restriction enzymes and T4-DNA ligase were obtained from Takara Shuzo, Toyobo, New England Biolabs (NEB), or United States Biochemicals (USB).

Mutagenesis—The plasmid pTZDHFR 20 contains an efficient expressive and reconstructed gene for the Cys85→Ala and Cys152→Ser double mutant DHFR (AS) (*2*). The N-terminal extension of the AS protein was carried out by cassette mutagenesis using unique *Bcl*I and *Nhe*I sites (Fig. 1). The C-terminal extension was effected using unique *Xho*I and *Bam*HI sites. The converted sequences and their abbreviations are shown in Fig. 1.

Digestion with restriction enzymes and ligation of DNAs were carried out according to the manufacturers' protocols. *E. coli* HB101 was used as a cloning host. For single-strand DNA preparation. *E. coli* JM109 was used with the aid of helper phage VCS-M13 (Stratagene). Competent cells of *E. coli* were prepared and transformed as described by Hanahan (*7*). Plasmids were prepared as described by Birnboim and Doly (*8*) or by Maniatis *et al.* (*9*). Nucleotide sequencing was carried out by the Sanger method using a single strand DNA as a template (*10, 11*).

Protein Purification and Enzyme Assay—Mutant DHFRs were purified mainly by MTX-affinity chromatography, taking advantage of adequate prepurification steps from cell-free extracts (*12*). Mutant DHFRs were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (*13*). From 20 g of wet cells, 100-200 mg of the purified protein was usually obtained. Protein concentration was determined from absorbance at 280 nm using the extinction coefficient of $\varepsilon_{280} = 3.11 \times 10^4\ M^{-1} \cdot cm^{-1}$ (*14*) for the wild-type DHFR. This value of ε_{280} was confirmed by measuring the protein concentration by the Bradford method (*15*). The activity of DHFR was determined spectrophotometrically at 25°C by following the disappearance of NADPH and DHF at 340 nm ($\varepsilon = 11{,}800\ M^{-1}$) (*16*). The assay mixture contained 50 μM DHF, 60 μM NADPH, 12 mM 2-mercaptoethanol, MTEN buffer, pH 7.0 (*17*), and the enzyme in a final volume of 1 ml. The mixture was degassed by aspiration before addition of the enzyme.

The enzymatic activity remaining after heat treatment was used as a measure of thermal reversibility. Protein samples (various concentrations in 0.1 ml) were incubated in 10 mM K-phosphate, pH 6.8, containing 0.2 mM EDTA at 80°C for 30 min, then chilled on ice for more than 20 min. For the reduced-form proteins, the buffer also contained 14 mM 2-mercaptoethanol to prevent air oxidation of Cys-SH residues. The remaining enzymatic activity was determined by using an adequate amount of the protein and the standard enzyme assay mixture without 2-mercaptoethanol.

Oxidation of Cys-SH—Oxidation with *trans*-4,5-dihydroxy-1,2-dithiane (oxidized DTT) of the introduced Cys-SH was carried out in 10 mM phosphate buffer, pH 7.0, containing 0.1 mM EDTA and oversaturated concentration (0.1 g/ml) of oxidized DTT overnight at room temperature at a protein concentration of 1 mg/ml with gentle stirring. After removing the insoluble oxidized DTT by centrifugation, 5,5′-dithio-bis(2-nitrobenzoate) (DTNB) solution (pH

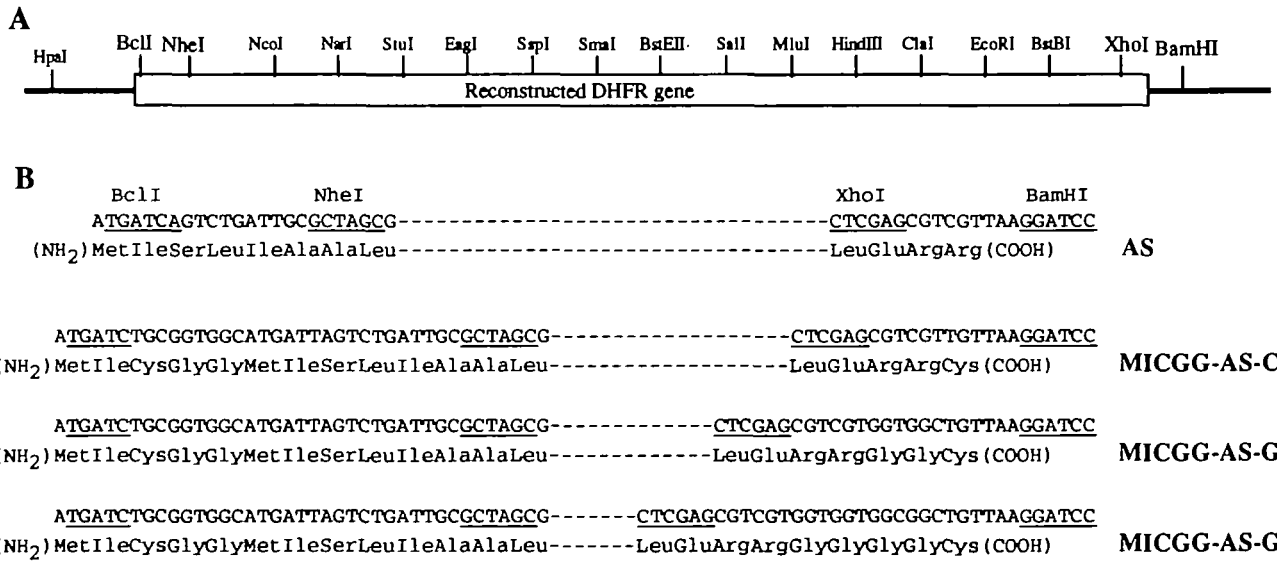

Fig. 1. **(A) Schematic representation of restriction sites in the reconstructed gene (540 bp from *Hpa*I site to *Bam*HI site) for Cys-free mutant DHFR (AS).** The reconstructed gene is carried on the plasmid pTZDHFR 20 (*2*). **(B) Cassette mutagenesis for N- and C-terminal extensions.** Synthetic DNAs shown in the figure were introduced to *Bcl*I-*Nhe*I sites for the N-terminal region, creating the 5-amino acid extension (MICGG), and *Xho*I-*Bam*HI sites for the C-terminal region, creating three types of extensions: C, GGC, and GGGGC. The amino acid sequences of the N- and C-terminal parts of the mutant proteins and their abbreviations are also shown.

8.0) was added with gentle stirring to give a final concentration of 0.5 mM. Unoxidized Cys-SH residues were thus modified by DTNB. The treated enzyme sample was dialyzed against 10 mM K-phosphate, pH 6.8, containing 0.2 mM EDTA, three times. The DHFR with an internal disulfide bond (circular DHFR) was purified by DEAE-Toyopearl column chromatography. Because the enzyme modified with DTNB had a greater negative charge than the circular DHFR, the separation of the two was complete.

CD Measurement—CD spectra were recorded from 250 to 200 nm on a JASCO J-600 spectrophotometer, which was calibrated with ammonium d-10-camphorsulfonate (*18*). The temperature of the protein sample was controlled with a thermostated cell housing. Each spectrum was obtained as the average of at least 4 scans, and represented as the mean residual molar ellipticity $[\theta]$.

Thermal denaturation of proteins was measured by monitoring the ellipticity at 222 nm as a function of temperature, which was raised at a constant rate of 1°C/min from 15 to 80°C, using a temperature-scanning CD system (*19*).

Prior to CD measurements, each protein was dialyzed three times against buffer which has been degassed by aspiration. The buffers used for the oxidized and reduced proteins were 10 mM K-phosphate buffer, pH 6.8, containing 0.2 mM EDTA and the same buffer containing 0.2 and 14 mM 2-mercaptoethanol, respectively.

HPLC Analysis—Gel-filtration HPLC analysis of heat-treated proteins was carried out on a SMART system or on a LKB Ultrochrom GTi HPLC system on a Superdex 75 or Superose HR12 prepacked column. The elution buffer was 10 mM potassium phosphate buffer, pH 6.8, containing 0.2 mM EDTA and 0.1 M KCl.

Others—N-Terminal amino acid sequence was examined by Edman degradation on a Beckman LF3000 protein sequencer equipped with an on-line PTH amino acid analyzer, System Gold. SDS-PAGE was carried out by the method of Laemmli (*13*). The polyacrylamide gradient gel (10–20%) was purchased from Daiichi Chemicals. The proteins were stained with Coomassie Blue. As molecular weight standard proteins, a Sigma Dalton Mark VII-L was used. Electro-spray mass spectra were taken by using a Perkin Elmer API-III mass spectrometer.

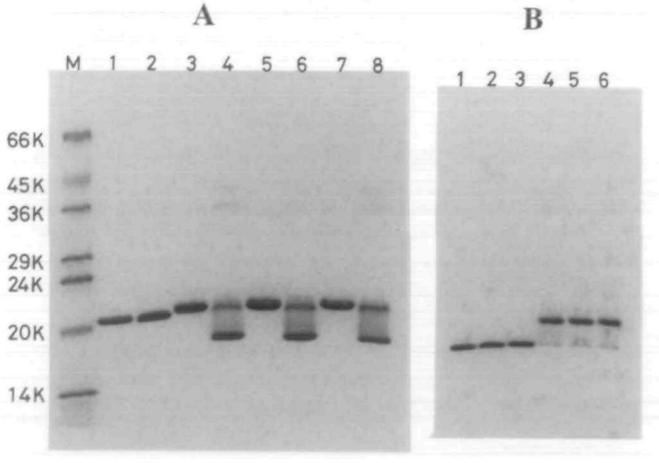

Fig. 2. **Nonreducing SDS-polyacrylamide gel electrophoresis.** (A) Oxidation of the mutant proteins by oxidized DTT. Lane 1, untreated AS; lane 2, oxidized AS; lane 3, untreated MICGG-AS-C; lane 4, oxidized MICGG-AS-C; lane 5, untreated MICGG-AS-G2C; lane 6, oxidized MICGG-AS-G2C; lane 7, untreated MICGG-AS-G4C, lane 8, oxidized MICGG-AS-G4C, lane M, molecular weight marker proteins. (B) Test for formation of intermolecular disulfide bond after measuring thermal transition by CD up to 80°C at protein concentration of 8.5 μM. Lane 1, oxidized form (o-) MICGG-AS-C; lane 2, o-MICGG-AS-G2C, lane 3, o-MICGG-AS-G4C; lane 4, reduced form (r-) MICGG-AS-C; lane 5, r-MICGG-AS-G2C, lane 6, r-MICGG-AS-G4C

RESULTS

E. coli DHFR has two free Cys residues at positions 85 and 152 and no intramolecular disulfide bond. A Cys-free mutant DHFR (AS, Cys85→Ala, Cys152→Ser double mutant) is as active as the wild-type enzyme and shows essentially the same folding kinetics and conformational stability (*2*). The N- and C-terminal extension was carried out by cassette mutagenesis and three AS derivatives, designated MICGG-AS-C, MICGG-AS-G2C, and MICGG-AS-G4C, were constructed (Fig. 1). These mutant proteins were highly purified by the procedure previously reported (*12*) and showed enzymatic activity as high as the wild-type enzyme (Table I).

Purified proteins were oxidized with the aid of oxidized DTT to give an extra loop connecting between the termini by a single disulfide bond. Because the oxidation reaction was not complete, as shown in Fig. 2A, the free SH groups of the unoxidized protein were treated with DTNB, and the modified protein was removed by DEAE-Toyopearl column chromatography.

Finally, a homogeneous sample of oxidized proteins was obtained. The formation of intermolecular disulfide bonds,

TABLE I. **Summary of the molecular properties of the reduced (r-) and oxidized (o-) forms of mutant DHFRs.**

Enzyme	Enzymatic activity (units/mol)[a]	Molecular weight		Thermal transition		Reversibility (%)	
		Calculated	Measured	T_m (°C)	ΔH (kcal/mol)	By CD[b]	By activity[c]
r-MICGG-AS-C	7×10^4	18,517 1	18,515.6	51.9	51 0	45	46
o-MICGG-AS-C	7×10^4	18,515.1	18,513.2	58 8	59.7	100	100
r-MICGG-AS-G2C	7×10^4	18,631 2	18,628 6	53 5	52.9	45	46
o-MICGG-AS-G2C	7×10^4	18,629.2	18,627.1	58.6	71.9	100	100
r-MICGG-AS-G4C	7×10^4	18,745 3	18,742 9	53.9	56.4	45	48
o-MICGG-AS-G4C	7×10^4	18,743.3	18,739.3	60.4	75.5	100	100
AS	7×10^4	17,952.3	17,951.6	55.2	53.9	95	88
Wild type	7×10^4	18,000.5	17,997.3	54.3	43.7	85	80

[a]At 25°C. [b]Recovery of CD value at 222 nm at protein concentration of 0.15 mg/ml, after thermal transition experiment as typically shown in Fig 4; $100\times[CD_{222}$ (cooled, at 15°C) $-$ CD_{222} (at 80°C)$]/[CD_{222}$ (native, at 15°C) $-$ CD_{222} (at 80°C)$]$. [c]Remaining DHFR activity after the thermal transition experiment

resulting in higher molecular weight protein bands around 36K-45K, was quite rare. Of the three mutant proteins, intermolecular disulfide bond formation was apparently suppressed in MICGG-AS-G2C, suggesting that it had the most favorable loop length, (C-terminus)-GGC-(disulfide bridge)-GG-(N-terminus).

All the oxidized (o-) forms of the mutant proteins with S-S loops of different sizes had enzymatic activity as high as wild-type DHFR (Table I). This may reflect the flexible nature of the terminal regions, which allows the distance between the N- and C-termini to vary so as to be compatible with the introduced S-S loops. The mass spectroscopic data indicated that there was no large modification in the proteins used in this study (Table I). The CD spectra of the reduced and oxidized proteins at low temperature (15°C, folded condition) coincided with each other (Fig. 3).

Moreover, there was no detectable difference between the CD spectra of the reduced and oxidized forms at high temperature (80°C, unfolded condition). Thus, the formation of the loop between N- and C-termini caused no serious perturbations in secondary structures in both native and denatured states. In contrast, the thermal denaturation curve of the engineered protein as obtained by CD measurement at protein concentration of 0.15 mg/ml (8.5 μM) at 222 nm was shifted to higher temperature by oxidation: namely, the formation of the extra loop between the N- and C-termini increased thermal stability, as typically shown in Fig. 4.

The temperatures at the transition midpoint, T_m, were 58.8, 58.6, and 60.4°C for the oxidized forms of MICGG-AS-C, MICGG-AS-G2C, and MICGG-AS-G4C, and 51.9, 53.5, and 53.9°C for the reduced counterparts, respectively (Table I). The transition enthalpies (ΔH) for the oxidized forms of MICGG-AS-C, MICGG-AS-G2C, and MICGG-AS-G4C were respectively 59.7, 71.9, and 75.5 kcal/mol, 8-9 kcal/mol higher than those for the reduced counterparts. The T_m and ΔH for the AS protein (without the extended termini) were 55.2°C and 53.9 kcal/mol, respec-

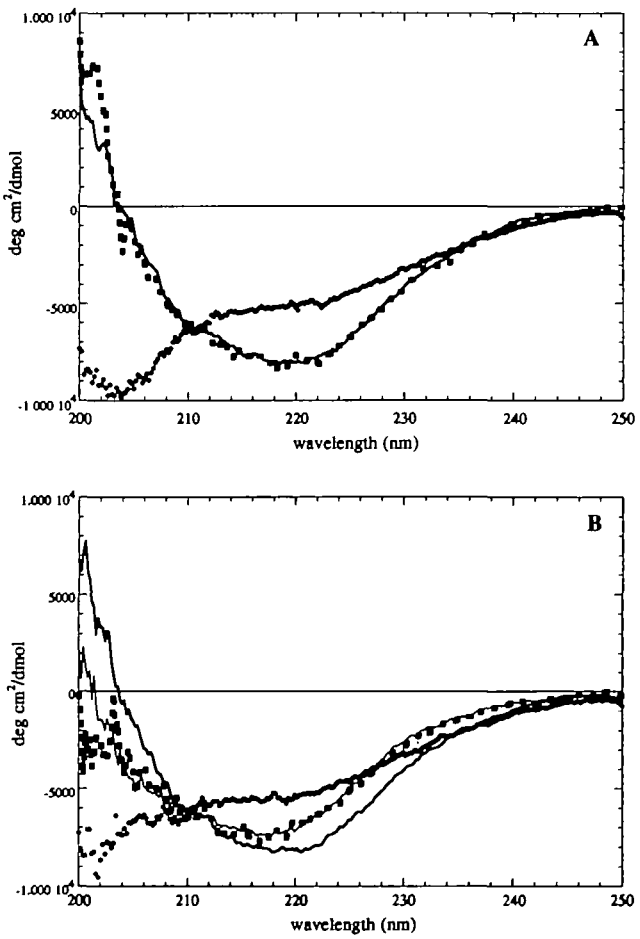

Fig. 3. **CD spectra of native, heat-denatured, and heat-treated proteins.** (A) CD spectra of o-MICGG-AS-G2C. —, native protein at 15°C; ∞∞, denatured proteins at 80°C; ▪▪▪▪, heat-treated protein, 5 min after cooling from 80 to 15°C. The protein concentration was 8.5 μM. (B) CD spectra of r-MICGG-AS-G2C. —, native protein at 15°C; ∞∞, denatured proteins at 80°C; ▪▪▪▪, heat-treated protein, 5 min after cooling from 80 to 15°C; — heat-treated protein, 6 h after cooling from 80 to 15°C. The protein concentration was 8.5 μM.

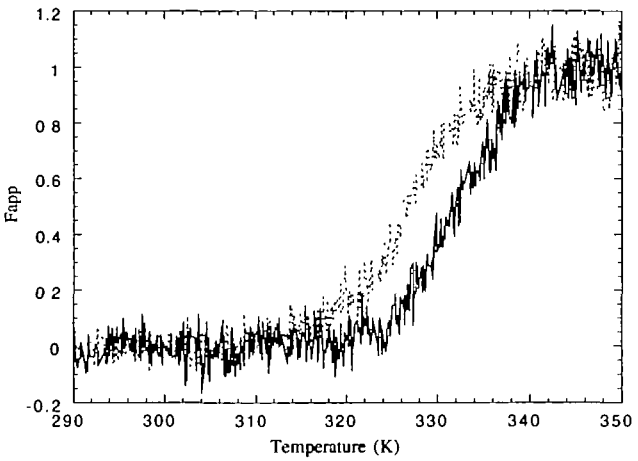

Fig. 4. **Heat denaturation of o- and r-forms of MICGG-AS-G2C protein.** The apparent molar fraction of denatured states was evaluated from CD data at 222 nm on the assumption of a two-state transition, N⟷D. —, o-MICGG-AS-G2C; ----, r-MICGG-AS-G2C. The protein concentration was 8.5 μM.

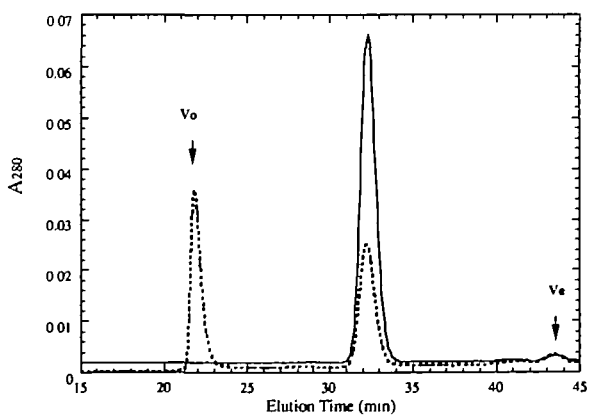

Fig. 5. **Gel-filtration HPLC analysis of heat treated proteins.** —, o-MICGG-AS-G2C; ····, r-MICGG-AS-G2C. V_o and V_e indicate the void volume and the elution position of low molecular compounds, respectively. HPLC was carried out on a SMART system on a Superdex 75 prepacked column (3.2×300 mm, exclusion molecular weight=100,000 and resolution range=3,000-70,000). 10-μl of protein sample (8.5 μM) was analyzed with flow rate of 40 μl/min.

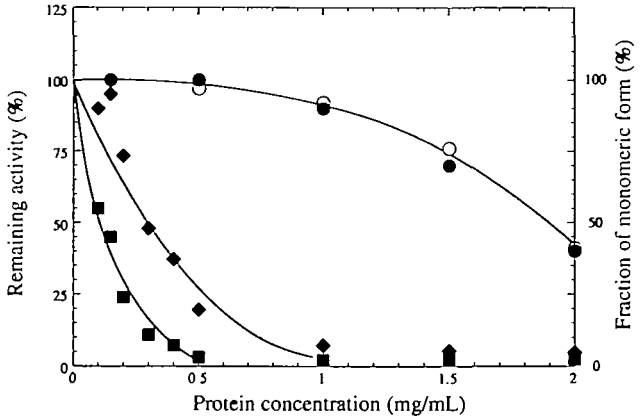

Fig. 6. **Effect of protein concentration on the reversibility of thermal denaturation.** The remaining enzymatic activity (%) after heat treatment of the protein at various protein concentrations at 80°C for 30 min was used as a measure of thermal reversibility; ●, o-MICGG-AS-G2C; ■, r-MICGG-AS-G2C; ◆, AS. For the o-MICGG-AS-G2C, ○, the relative fraction (%) of monomeric form using the same sample of the heat-treated protein was determined by gel-filtration HPLC (LKB Ultrochrom GTi) on a Superdex 75 prepacked column and is plotted as a function of protein concentration.

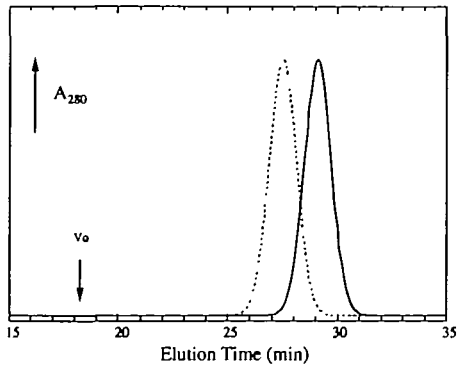

Fig. 7. **Gel-filtration HPLC analysis of the denatured proteins in the presence of 5 M guanidine-HCl: —, o-MICGG-AS-G2C; ···· , r-MICGG-AS-G2C.** HPLC was carried out on an LKB Ultrochrom GTi system on a Superose HR12 10/30 prepacked column using 10 mM K-phosphate buffer, pH 6.8, containing 5 M guanidine-HCl, 0.2 mM EDTA as a elution buffer. A 100-μl sample of protein (30 μM in the elution buffer) was applied to the column and eluted at a flow rate of 0.4 ml/min. V_o indicates the void volume.

tively. These values for the wild type DHFR were 54.3°C and 43.7 kcal/mol.

The reversibility of the reduced protein at the low protein concentration of 0.15 mg/ml was not complete even after long incubation of the heated proteins at 15°C (Fig. 3B). As much as 55% of the heat-treated r-MICGG-AS-G2C was eluted as a high molecular aggregated form, while the remaining protein was eluted at the same positions as the o-MICGG-AS-G2C (Fig. 5) and as the native r-MICGG-AS-G2C (data not shown).

The fraction of the unaggregated form coincided with both the recovery of CD signal (45%) and the remaining enzymatic activity (46%) (Table I). The loss of the thermal reversibility as measured by the enzymatic activity was dependent on protein concentration (Fig. 6).

Almost all of the activity of the reduced form was lost at the protein concentration as low as 0.5 mg/ml. The SDS-PAGE in the absence of the reducing agent, 2-mercaptoethanol, showed that only a small amount of high molecular compounds was present in the protein samples after the thermal denaturation (Fig. 2B). This strongly suggests that noncovalent interaction is the major factor in the aggregation rather than intermolecular disulfide bonding. The reversibility of the AS protein (part of the MICGG-AS-G2C) was not complete at the low protein concentration of 0.15 mg/ml, and 80% of the activity was lost at the protein concentration of 0.5 mg/ml. On the contrary, the thermal transition of the oxidized proteins at the low protein concentration of 0.15 mg/ml was completely reversible: the CD spectra were completely recovered within 5 min after cooling to 15°C and showed the native form (Fig. 3A); there was no loss of the enzymatic activity in the cooled sample (Table I); and no aggregated materials could be detected in heat-treated o-MICGG-AS-G2C sample on gel-filtration HPLC (Fig. 5). More than 90% of the enzymatic activity was recovered after heat treatment at 80°C for 30 min at protein concentrations up to 1.0 mg/ml. Also, the loss of the enzymatic activity in the oxidized form was coincident with the formation of aggregated materials at the higher protein concentrations (Fig. 6).

As shown in Fig. 2, the oxidized and reduced proteins could be separated by SDS-PAGE in the absence of reducing agent. This may reflect the difference in the denatured structures of the proteins: an extended structure in the reduced proteins (slower mobility) and a compact structure retained in the oxidized proteins (faster mobility). Similarly, both proteins were completely separable by gel-filtration HPLC in the presence of 5 M guanidine-HCl (Fig. 7), in contrast to the native condition where the oxidized and reduced proteins eluted at essentially the same time.

The earlier elution of the reduced proteins reflects the extended denatured structure as compared with the counterpart oxidized proteins, which eluted later.

DISCUSSION

Our results presented here clearly demonstrate that the reversibility of thermal denaturation and the thermal stability itself are greatly improved by covalently connecting the N- and C-termini of dihydrofolate reductase through an extra loop containing a disulfide bond (Fig. 8).

Protein concentration-dependent intermolecular association is a major reason for the irreversibility as demonstrated in Figs. 5 and 6, although virtually no aggregate is present in highly concentrated DHFR samples (more than 20 mg/ml) in the native condition (*20*). This association probably takes place during the refolding process. The refolding reaction of urea-induced unfolded DHFR, which is completely reversible, in contrast to thermal denaturation, is specifically inhibited by the addition of proteolytic fragments of DHFR (although in 1,000-fold excess) (*21*). The stabilized DHFR with an engineered disulfide bond inside the molecule (positions 39 and 85) showed less thermal reversibility than the wild-type enzyme (*5*). This suggests that the terminal region is a key to intermolecular association: a decrease in the apparent volume of the denatured proteins due to the connection of the termini may prevent intermolecular interaction, which may be the

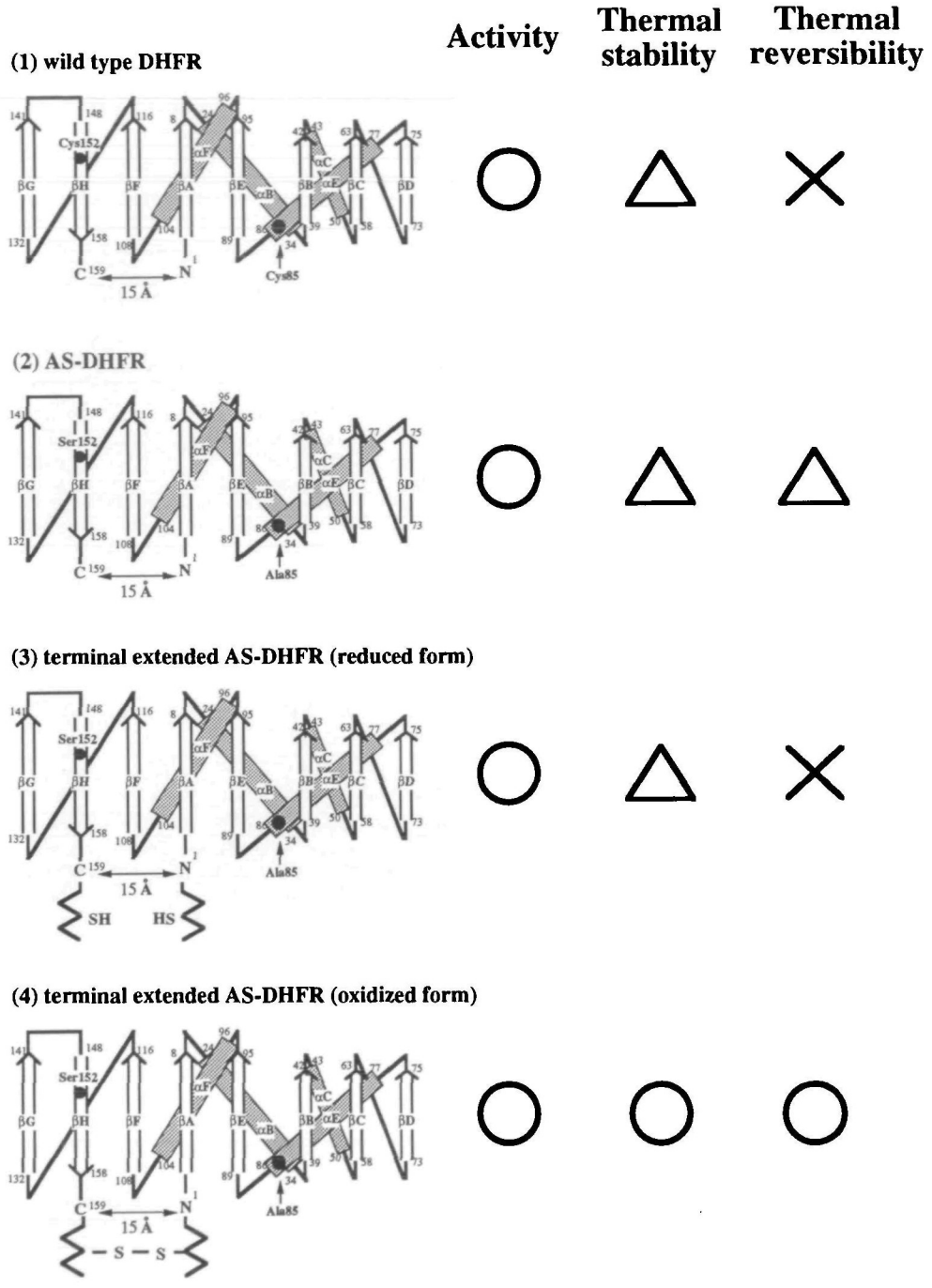

Fig. 8. **Schematic drawing of the secondary structure arrangement and comparison of activity, thermal stability, and thermal reversibility of the wild-type DHFR (1), AS-DHFR (2), reduced form (3), and oxidized form (4) of terminal extended AS-DHFR.** ○, △, and × indicate good, moderate, and bad, respectively.

result of interaction between exposed terminal regions or be triggered by the presence of an exposed terminal region. A limitation of spatial movement of the terminal regions prevents such interactions and, therefore, the intermolecular irreversible aggregation. The thermal reversibility of T4 lysozyme is improved by engineering a 3-97 disulfide bond (but not 54-97 bond), and this is indeed consistent with reduced mobility of the N-terminal region by the disulfide bond (*22*).

Many works have been performed to introduce cross-links inside protein structure with the aim of reducing a local conformational flexibility and thereby improving protein stability. However, they showed mixed results (*23*): stabilization, no effect, or destabilization. Protein stability can be enhanced by covalent cross-linking that limits the conformational freedom of the unfolded chain, if the cross-linkage itself does not change the local interactions (*24*). This entropic effect on the stabilization is increased with increasing size of the loop formed by the cross-link (*24, 25*). Thus, a connection between the N- and C-termini through an extra loop should provide a better entropic effect than any other intramolecular cross-link. Moreover, the perturbation of the local interaction at the terminal region may be minimized by changing the Gly-spacer to adjust the distance between the N- and C-termini. Thus, the strategy described in this paper will be applicable

to other monomeric proteins whose termini are in close proximity, and thermal stability and reversibility may also be improved by limiting terminal flexibility.

Reversible control of an enzymatic reaction by physical means such as temperature shift is attractive in practical uses of protein catalysts that require for on-off switching of the reaction. However, thermal denaturation of many proteins often causes irreversible aggregation and inactivation. As shown in this paper, the problem of thermal irreversibility can be resolved (although not completely) by limiting the terminal flexibility. Then, it becomes possible to set up an enzymatic reaction system with reversible on-off control. Such approaches are now underway.

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