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Cyanocysteine-Mediated Molecular Dissection of Dihydrofolate Reductase: Occurrence of Intra- and Inter-Molecular Reactions Forming a Peptide Bond

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During a cyanocysteine-mediated dissection study of dihydrofolate reductase, a peptide fragment with a molecular mass of 18 Da less than expected was found as a major reaction product when the dissection reaction was applied to a Lys-cyanocysteine linkage. Detailed characterization of the dissection products by protease digestion, peptide sequencing, liquid chromatography/electrospray ionization mass spectrometry, and capillary electrophoresis suggested that the by-product was generated *via* a lactam ring formation through the intramolecular nucleophilic attack of the ε-amino group on the carbonyl carbon of the Lys-cyanocysteine linkage. We have also demonstrated the occurrence of intermolecular attack of an α-amino group of glycine on the carbonyl carbon of the X-cyanocysteine linkage to form a new X-Gly linkage, which should be a useful reaction for specific modification of proteins at the C-terminal.

Key words: cyanocysteine, dihydrofolate reductase, mass spectrometry, molecular dissection, peptide bond.

Molecular dissection of proteins is increasingly being employed to analyze their functional and structural units (*1*). There are many ways to generate protein fragments, each with inherent advantages and disadvantages. Enzymatic proteolysis of a purified protein can be used conveniently to generate protein fragments, but multiple cleavage sites with variable reactivities can make the production of the desired protein fragments difficult. Recombinant techniques have been used to produce protein fragments by inserting termination codons at predetermined locations, but the yields of marginally stable fragments are often compromised. Chemical cleavage methods can also provide access to large fragments, but are often associated with undesirable side reactions. Chemical cleavage at cysteine residues, namely, *S*-cyanocysteine-mediated dissection reaction, is particularly attractive because of the rather distinct reactivity and the scarcity of free cysteine in many proteins. As extensively studied by Catsimpoolas and Wood (*2*) and Jacobson *et al.* (*3*), the cleavage reaction of the peptide bond at cyanocysteine residues (*3*), which can be formed after cyanidation of SH groups by 2-nitro-5-thiocyanobenzoic acid (NTCB) (*4, 5*), is a highly specific and mild reaction. As shown in Fig. 1, this chemical cleavage reaction generates an N-terminal peptide with a free C-terminal as well as a C-terminal peptide with a 2-iminothiazolidine-4-carboxyl (ITC) N-terminal (route 1). The ITC N-terminal of the C-terminal peptide can be converted to alanine by catalytic reduction (*6*). The cyanocysteine residue is also converted to a dehydroalanine residue by a β-elimination reaction (route 2), which decreases the yield of the dissection reaction. No other reaction routes than the 2-pyrrolidone-5-carboxylic acid formation from *S*-cyanoglutathione (*3*) had been reported in the *S*-cyanocysteine-mediated dissection reaction before this work was started.

Dihydrofolate reductase (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 (*7*). Because of its crucial role in metabolism, relatively small size (18 kDa), and monomeric structure, DHFR has become the subject of a variety of biochemical, biophysical, and protein engineering studies (*8-12*). The modification-assisted site-specific dissection approach has been applied to DHFR to find a stable peptide fragment in DHFR sequence. For this purpose, unique cysteine residues have been introduced at various positions in a Cys-free mutant DHFR (*14*) by site-directed mutagenesis, and the resulting engineered DHFRs have been

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Abbreviations: Lys-EP, *Achromobacter* protease I (lysine-specific endopeptidase); CE, capillary electrophoresis; DTT, dithiothreitol; DHFR, dihydrofolate reductase; Gu-HCl, guanidine hydrochloride; ITC, 2-iminothiazolidine-4-carboxyl; LC/MS, liquid chromatography/mass spectrometry; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; LC/ESI-MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; MS, mass spectrometry; NTCB, 2-nitro-5-thiocyanobenzoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MS/MS, tandem mass spectrometry; Ser77→Cys mutant DHFR, triple mutant of dihydrofolate reductase with Cys85→Ala, Cys152→Ser, and Ser77→Cys replacements; Tyr111→Cys mutant DHFR, a triple mutant of dihydrofolate reductase with Cys85→Ala, Cys152→Ser, and Tyr111→Cys replacements.

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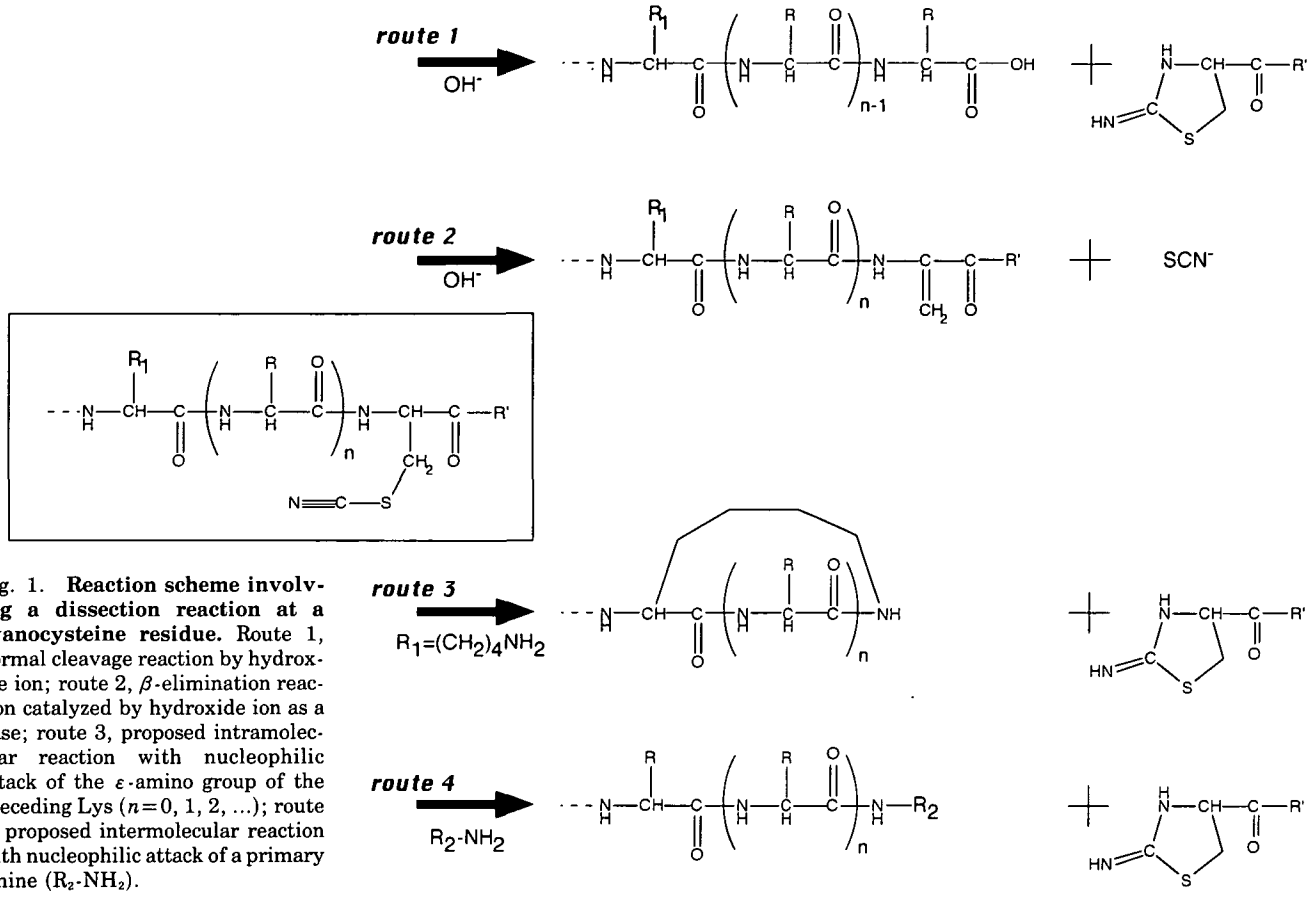

Fig. 1. **Reaction scheme involving a dissection reaction at a cyanocysteine residue.** Route 1, normal cleavage reaction by hydroxide ion; route 2, β-elimination reaction catalyzed by hydroxide ion as a base; route 3, proposed intramolecular reaction with nucleophilic attack of the ε-amino group of the preceding Lys ($n=0, 1, 2, \ldots$); route 4, proposed intermolecular reaction with nucleophilic attack of a primary amine (R_2-NH_2).

subjected to *S*-cyanocysteine-mediated dissection reaction. During these studies, we monitored the reaction products using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS), and observed the formation of by-products when the dissection reaction was applied to a Lys-cyanocysteine linkage. The molecular mass characterization of the isolated cleaved fragment by reverse phase HPLC showed a by-product peptide with a molecular mass of 18 Da less than expected. Characterization of the peptide fragment by protease digestion, liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), and capillary electrophoresis (CE) suggested that the 18 Da loss is related to lactam ring formation by the nucleophilic attack of the ε-amino group of the lysine residue on the carbonyl carbon of the X-cyanocysteine linkage in the molecular dissection reaction (route 3). Based on the proposed reaction (route 4), we have demonstrated the occurrence of intermolecular attack on the carbonyl carbon of the X-cyanocysteine linkage by the α-amino group of glycine. This should be useful for C-terminal modification of proteins, such as C-terminal extension reaction.

MATERIALS AND METHODS

Chemicals—Methotrexate (MTX)-agarose affinity resin was obtained from Sigma. DEAE-Toyopearl 650M was from Tosoh (Tokyo). Restriction enzymes, T4-DNA ligase, Taq polymerase, *Achromobacter* protease I (Lys-EP), *Staphylococcus aureus* V8 protease (V8), and trypsin were obtained from Takara Shuzo (Kyoto). All other chemicals were of reagent grade.

Plasmid Construction and Protein Purification—The cysteine-free DHFR has been constructed previously (*13*). Site-directed mutagenesis at Ser77 and Tyr111 was carried out by PCR methods (*14*).

Protein purification was carried out mainly by means of methotrexate bound affinity chromatography after appropriate pre-purification steps from cell-free extracts (*15*). The purified proteins were homogenous as judged by SDS-polyacrylamide gel electrophoresis (*16*) and were stored in saturated ammonium sulfate solution as precipitates at 4°C until use. Protein concentrations of wild-type and Ser77→Cys mutant DHFRs were determined from the absorbance at 280 nm using the molar extinction coefficient ($\varepsilon_{280}=31{,}100\ M^{-1}\cdot cm^{-1}$) (*17*) for the wild-type DHFR. No detectable change in extinction coefficient was observed for Ser77→Cys mutant DHFR when ε_{280} was determined by measuring the protein concentration by the Bradford method (*18*) using the wild-type DHFR as a standard. Protein concentration of the Tyr111→Cys mutant DHFR was determined by the Bradford method. The activity of DHFR was determined spectrophotometrically at 15°C following the disappearance of NADPH and DHF at 340 nm ($\varepsilon_{340}=11{,}800\ M^{-1}\cdot cm^{-1}$) (*19*). The assay mixture contained 50 μM DHF, 60 μM NADPH, 14 mM 2-mercaptoethanol, MTEN buffer [50 mM 2-morpholinoethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM NaCl], pH 7.0 (*20*), and the enzyme in a final volume of 1 ml.

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Cyanidation and Cleavage of Proteins—Precipitated (stored) protein was collected by centrifugation at 20,000 × *g* for 30 min. The collected protein was dissolved in small volume of Tris buffer (0.1 M Tris-HCl, pH 7.4, containing 5 mM EDTA) to make a protein concentration of *ca.* 0.3 mM and dialyzed three times against the same buffer. Prior to the chemical modification, protein was treated with 1 mM DTT at room temperature for at least 2 h, and then dialyzed against 0.1 M Tris-HCl, pH 7.4, containing 5 mM EDTA. The dialyzed protein was further purified by reverse phase HPLC. The protein thus obtained was lyophilized and then dissolved with 0.1 M Tris-HCl, pH 7.4, containing 6 M guanidine-hydrochloride (Gu-HCl) and 5 mM EDTA. About 0.1 mM DHFR-mutant protein containing a unique Cys-residue was treated with a 5-fold molar excess of 2-nitro-5-thiocyanobenzoic acid (NTCB) in the same buffer for 4 h at 37°C. Then, the modified protein was purified by reverse phase HPLC (Waters HPLC system and YMC D-ODS-5 (ϕ20 mm × 250 mm) column) with a linear gradient of acetonitrile from 35% to 55% in 0.1% TFA. The modified protein thus obtained was lyophilized and then dissolved in 0.1 M Tris-HCl, pH 9.3, in the presence of 6 M Gu-HCl and 5 mM EDTA. The cleavage reaction was completed by incubation in the same buffer, and resulted in the N-terminal fragment, C-terminal fragment, and dehydroalanine derivative of DHFR. The reaction products were monitored by LC/ESI-MS.

LC-MS Measurements—All mass-spectroscopic (MS) measurements were carried out in the positive-ion mode using a PE Sciex API III triple quadrupole mass spectrometer. For on-line LC/MS measurements the nebulization probe inlet was coupled directly to a splitting device. The split was adjusted to provide a flow of 5 μl/min to the mass spectrometer (total effluent, 200 μl/min). Separation was carried out with an acetonitrile gradient from 10% (0 min) to 60% (30 min) containing 0.1% TFA for L-column ODS (ϕ2.1 × 150 mm; Kagaku-hin Kensa Kyoukai, Tokyo). The ionspray tip was held at a potential of 5,000 V. Compressed nitrogen (pressure set at 5.7 kg/cm^2 and flow-rate at 0.8 liter/min) was employed to assist liquid nebulization. A curtain gas (nitrogen) flow of 1.2 liters/min was used to prevent moisture from reaching the orifice and the quadrupole guidance lens. The MS system was operated with an orifice voltage of 100 V and the scanning range was m/z from 400 to 2,400 using a step size of 0.5 unit and a dwell time of 1 ms. Spectra were obtained as the averaged sum of 20 scans from 50 to 700 using dwell times of 10 ms per step of 1 Da with argon as the CID gas.

Sequencing—The 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. More than 100 pmol of peptide or protein samples was used for the analysis.

CE Measurements—CE was performed with Beckman PACE 2100. An uncoated fused-silica capillary (GL Sciences, Tokyo) of 50 μm i.d. and 25 cm length (18 cm to the detector), which was thermostated with a liquid coolant, was employed in all experiments. Detection was done by measuring absorbance at 214 nm. The sample was injected under pressure (0.5 kg/cm^2) for 2.0 s. The applied voltage was 8 kV. The capillary was rinsed with 1 N sodium hydroxide under pressure (1 min) after every run. All separation solutions were prepared with 0.05 of the ionic strength as reported previously (*21*). The electrophoretic mobility, μ, was measured as follows:

$$\mu = L(1/t_s - 1/t_0)l/V$$

where t_s and t_0 are the migration times of solute and the electroosmosis, respectively, L and l are the total capillary length and effective length, respectively, and V is the applied voltage. The charge of the solute, q, was calculated by using the equation according to Rickard *et al.* (*22*) as follows:

$$q = (\mu + 0.165)/(70.8\ M^{2/3})$$

where M is the molecular weight of the solute.

CD Measurements—CD spectra of the isolated protein fragments were recorded from 260 to 200 nm using an optical cell with a 1 mm light path on a JASCO J-600 spectrophotometer, which was calibrated with ammonium *d*-10-camphorsulfonate. The concentration of protein fragments was determined by measuring the absorbance at 280 nm using molar extinction coefficients estimated by the method of Gill and von Hippel (*23*). Samples (4–10 μM) were dissolved in 10 mM potassium phosphate, pH 7.0, containing 0.2 mM EDTA. The temperature of the 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.

Others—Digestion with restriction enzymes and ligation of DNAs were carried out according to the manufacturer's protocols. *Escherichia coli* HB101 was used as a cloning host. Preparation of competent cells of *E. coli* and transformation were performed as described by Hanahan (*24*). Preparation of plasmids was carried out according to the method described by Birnboim and Doly (*25*) or Maniatis *et al.* (*26*). 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 kit was used. The cleavage reactions with Lys-EP, V8 protease, and trypsin were carried out by the use of the protocols suggested by the manufacturers.

RESULTS

Site-Directed Mutagenesis to Introduce a Unique Cys Residue into Cys-Free DHFR—A unique cysteine residue was introduced at various positions of Cys-free DHFR (*13*) by site-directed mutagenesis, and the resulting engineered DHFRs were used for the chemical modification-assisted dissection.

Two positions (Ser77 and Tyr111: Fig. 2) were used in this study: Ser77 is preceded by Lys76, being located almost in the middle of the primary sequence, and is the starting amino acid of the α-helix E; Tyr111 is preceded by Lys109-Leu110, being located in the βF-strand, and the upstream N-terminal sequence contains all the α-helices. The Cys replacement at these positions did not essentially affect the DHFR enzymatic activity: k_{cat} values of wild type, Ser77→Cys DHFR, and Tyr111→Cys DHFR were 3×10^2, 3×10^2, and 1×10^2 min^{-1}, respectively; K_m (dihydrofolate) values of wild type, Ser77→Cys DHFR, and Tyr111→Cys DHFR were 1, 1, and 3 μM, respectively. The observed masses of Ser77→Cys DHFR and Tyr111→Cys DHFR were 17,966 and 17,890 Da, which are coinci-

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dent with calculated average masses of 17,967 and 17,892, respectively.

Dissection Reaction at Lys76-Cys77—As shown in Fig. 3A, Ser77→Cys DHFR even after reduction with DTT contained a small amount of materials, one of which had a molecular mass of 16 Da more than that of Ser77→Cys DHFR (data not shown), and is probably a product of oxidation of a Met residue during the storage in saturated ammonium sulfate solution. These contaminants could be removed by reverse phase HPLC, and the purified protein (Fig. 3A; peak a) was homogenous in terms of molecular mass. The isolated protein used for chemical modification with NTCB. The modified protein (cyanocysteine77-DHFR) was also purified by reverse phase HPLC (Fig. 3B; peak **b**), because, even though the reaction time for the modification was limited to 4 h to reduce the dissection reaction during the modification, minor fragment peaks were observed in addition to unmodified protein. The dissection of purified cyanocysteine77-DHFR was carried out in 0.1 M Tris-HCl, pH 9.3, containing 6M Gu-HCl and 5 mM EDTA, overnight at room temperature. The HPLC pattern of the resulting products is shown in Fig. 3C. Two major peaks with observed masses of 8,548 Da (peak **e**) and 9,443 (peak **c**) and two minor peaks with observed masses of 8,564 Da (peak **d**) and 17,936 Da (peak **f**) appeared. Peptide sequence analysis (10 cycles) of these peak samples showed that peaks **a, b, d, e**, and **f** had the same N-terminal sequence as that of wild type DHFR, while peak **c** did not show any sequence, suggesting that the N-terminal of the peak **c** peptide was blocked. Based on the mass number and

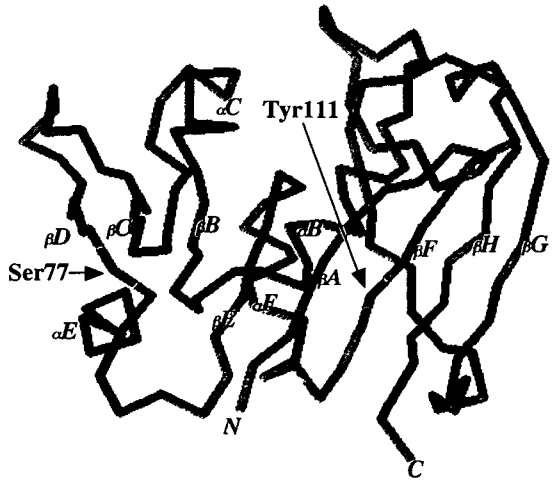

Fig. 2. **Structural representation of the backbone of DHFR [Bolin *et al.* (*29*)] showing the Ser77 and Tyr111 positions, which were the targets for the site-directed mutagenesis to Cys.** The secondary structural units are also represented.

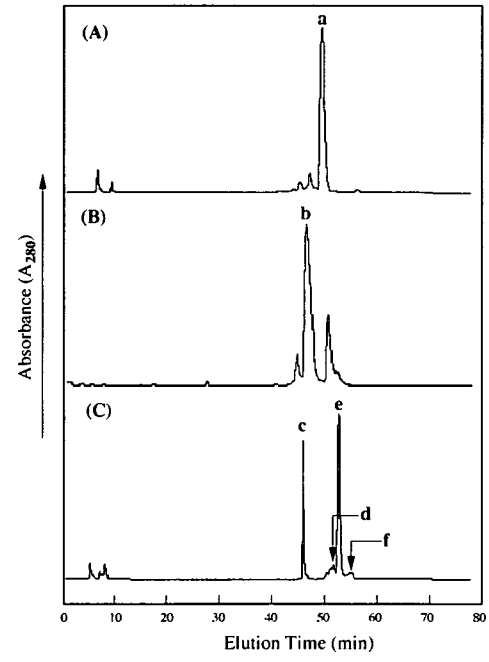

Fig. 3. **HPLC separation of modified and dissected Ser77→Cys DHFR.** (A) protein (total *ca.* 2 mg) reduced with DTT was applied to the YMC D-ODS-5 column with a linear gradient of acetonitrile from 35% (0 min) to 55% (60 min); (B) protein (total *ca.* 5 mg) modified with NTCB at pH 7.4 was applied to the YMC D-ODS-5 column with a linear gradient of acetonitrile from 35% (0 min) to 55% (60 min); (C) a dissected protein (total *ca.* 4 mg) after pH shift to pH 9.3 was applied to the YMC D-ODS-5 column with a linear gradient of acetonitrile from 10% (0 min) to 60% (60 min). The labeled peaks, **a, b, c, d**, and **e**, were isolated and subjected to ESI-MS measurement. The observed mass values summarized in Table I.

TABLE I. **Summary of molecular mass measurements and N-terminal sequences of Ser77→Cys DHFR and its derivatives.**

Protein or peptide	Molecular mass (Da)		N-terminal sequence		Assignment
	Measured	Calculated	From DNA Seq.	Experimental	
S-Cyanocysteine mediated dissection					
Peak **a** in Fig. 3A	17,966	17,967	MISILAALAV	MISILAALAV	Ser77→Cys DHFR
Peak **b** in Fig. 3B	17,993	17,992	MISILAALAV	MISILAALAV	Cyanocysteine77 DHFR
Peak **c** in Fig. 3C	9,443	9,444	CVDEAIAAG	Not detected	77-159 fragment with ITC N-terminal
Peak **d** in Fig. 3C	8,564	8,566	MISILAALAV	MISILAALAV	1-76 fragment
Peak **e** in Fig. 3C	8,548	8,566-18	MISILAALAV	MISILAALAV	1-76 fragment −18 Da (1-76′ fragment)
Peak **f** in Fig. 3C	17,936	17,936	MISILAALAV	MISILAALAV	Dehydroalanine77 DHFR
Lys-EP digestion of the peak **e** (1-76′ fragment) peptide					
Fragment 1	3,558	3,556	MISILAALAV	MISILAALAV	1-32 fragment
Fragment 2	744	744	RNTLNKPVIM	RNT	33-38 fragment
Fragment 3	2,288	2,284	PVIMGRHTWE	PVIMGR	39-58 fragment
Fragment 4	2,011	2,029-18	NIILSSQPGT	NIILSSQ	59-76 fragment −18 Da (59-76′ fragment)
Trypsin digestion of the fragment 4 (59-76′ fragment)					
Fragment 5	1,415	1,415	NIILSSQPGT	NIILSSQ	59-71 fragment
Fragment 6	614	632-18	VTWVK	VTW	72-76 fragment −18 Da (72-76′ fragment)

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N-terminal sequence, all the peaks except peak **e** could be assigned as follows: peak **a**, intact Ser77→Cys DHFR; **b**, cyanocysteine77-DHFR; **c**, 77-159 fragment of Ser77→Cys DHFR with 2-iminothiazolidine-4-carboxyl (ITC) derivative at the N-terminal (C-terminal fragment of normal dissected product); **d**, 1-76 fragment of Ser77→Cys DHFR (N-terminal fragment of normal dissected product); **f**, dehydroalanine derivative of Ser77→Cys DHFR (product of β-elimination reaction). Because the amount of the normal N-terminal fragment (peak **d**) was apparently less than that of the counterpart C-terminal fragment (peak **c**) and because the mass number of the peak **e** peptide (observed mass=8,548 Da) was smaller by 18 Da than that of normal N-terminal fragment (expected mass=8,566 Da), the peak **e** peptide was considered to correspond to the 1-76 fragment of Ser77→Cys DHFR but with some modification. Because four Trp residues out of five are present in the N-terminal half of the DHFR sequence (Trp22, Trp30, Trp47, Trp74, and Trp133), the height of peak **e** was higher than that of peak **c**. To identify the modification, the peak **e** peptide was further characterized by protease digestion and LC/ESI-MS. Table I summarizes the identification of the resulting peptides. The Lys-EP digestion revealed that the peptide fragment (59-76′ fragment) corresponding to residues 59-76 of the whole DHFR had a molecular mass of 18 Da less than expected (observed mass=2,011 Da, expected mass=2,029 Da). The tryptic digestion of the 59-76′ fragment showed that the peptide with the 59-71 sequence had a molecular mass consistent with the expected value (mass=1,415 Da), while there was a mass difference of 18 Da between the observed mass (=614 Da, 72-76′ peptide) and expected mass (=632 Da) of the fragment corresponding to 72-76. Examination of the 72-76′ peptide by MS/MS revealed that there were no y″ ions, but y″-18 ions were observed (Fig. 4). This indicates that loss of the 18 Da was due to the modification of the lysine residue at the C-terminal of the isolated fragment. Based on these results, the modification should be a lactam ring formation between the α-COOH and ε-NH_2 groups of the Lys76 residue, the C-terminal of the 72-76 sequence. The results of the following capillary electrophoretic (CE) study supported this assumption. The authentic 72-76 peptide (prepared by tryptic digestion of the original DHFR protein) and the 72-76′ peptide were used for the CE experiments. The electrophoregram at pH 2.7 is shown in Fig. 5A. Each peak was assigned by the injection of the individual sample. From the migration

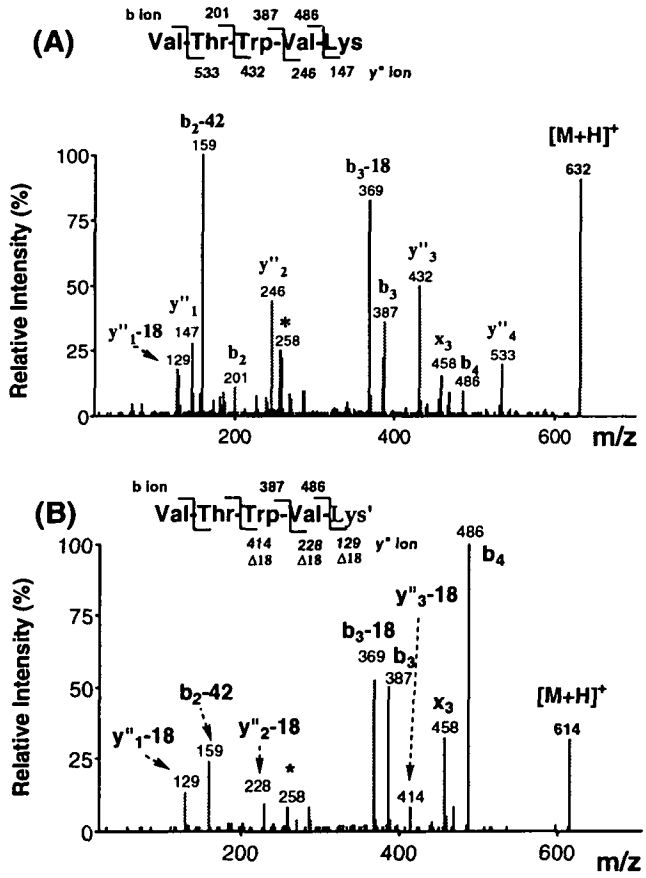

Fig. 4. **(A) Product ion spectra formed by CID of protonated ions for fragment molecules, an authentic 72-76 peptide with m/z=632 as a precursor ion (VTWVK). (B) Product ion spectra formed by CID of protonated ions for fragment molecules, a modified 72-76′ peptide with m/z=614 as a precursor ion (VTWVK′).** The product ions are labeled according to the nomenclature of Biemann (*30*).

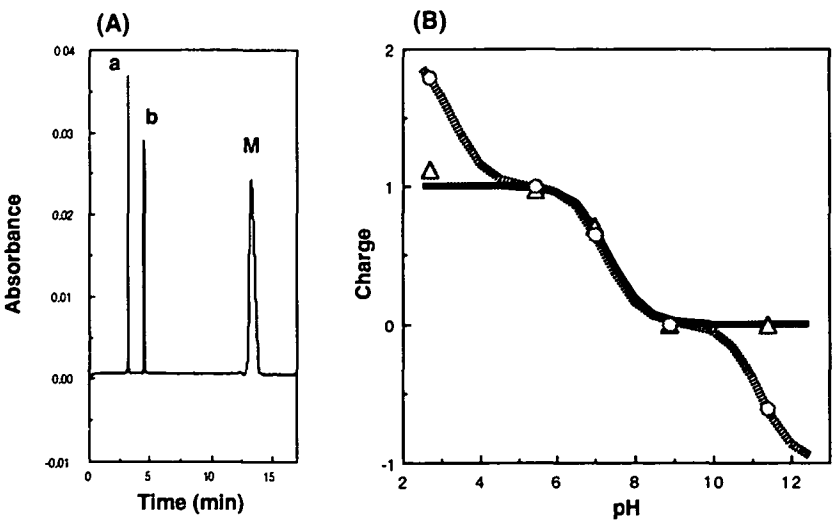

Fig. 5. **(A) A typical electrophoregram of peptides at pH 2.7.** Peak a, authentic 72-76 peptide; peak b, modified 72-76′ peptide; M, marker. **(B) pH-dependence of the charge of the authentic 72-76 peptide (○) and the modified 72-76′ peptide (△).** Determination of charge at various pH values was done as described in "MATERIALS AND METHODS." The authentic 72-76 peptide and the modified 72-76′ peptide were best described by the following Eqs. 1 and 2 (*21*), respectively:

[1] $q=\{2[H^+]^3+K_{a2}[H^+]^2-K_{a1}K_{a2}K_{a3}\}/\{[H^+]^3+K_{a3}[H^+]^2+K_{a2}K_{a3}[H^+]+K_{a1}K_{a2}K_{a3}\}$

[2] $q=[H^+]/(K_{a1}+[H^+])$

where K_{a1}, K_{a2}, and K_{a3} are the dissociation constants.

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times of the peptides, their charges were estimated by the method of Rickard *et al.* (*22*). Figure 5B shows the pH dependence of the charge of these peptides. The 72-76′ peptide was best described in terms of one ionic group with a pK_a value of 7.37 at the observed pH range (pH 2-12). Because the N-terminal of the 72-76′ peptide was free with respect to Edman degradation, the ionic group in this peptide must correspond to the α-amino group. On the other hand, the authentic 72-76 peptide [H_2N-Val72-Thr73-Trp74-Val75-Lys76(εNH_2)-COOH] was best described in terms of three ionic groups with pK_a values of 3.28, 7.27, and 11.21, probably corresponding to the α-carboxyl, α-amino, and ε-amino groups, respectively.

Dissection Reaction at Lys109-Leu110-Cys111—The dissection at Leu110-cyanocysteine111 of Tyr111→Cys DHFR was also carried out in the same manner as above. As dissection reaction products, we observed four major products with molecular mass of 17,857, 12,120, 12,103, and 5,812, which corresponded to the dehydroalanine derivative (expected mass = 17,851 Da), the normal N-terminal fragment (expected mass = 12,121 Da), modified N-terminal fragment (expected mass = 12,121-18 Da), and C-terminal fragment of the normal dissected product (expected mass = 5,812) as summarized in Table II. Thus, the 18 Da decrease in mass in the N-terminal fragment was also found in the molecular dissection at position 111 of the Tyr111→Cys DHFR. Digestion of the peptide fragment having a molecular mass of 12,103 Da by V8 protease yielded five peptide fragments corresponding to 1-48 (5,489 Da with the N-terminal sequence of Met-Ile-Ser-Ile-Leu), 49-80 (3,520 Da with the N-terminal sequence of Ser-Ile-Gly-Pro), 81-90 (913 Da with the N-terminal sequence of Ala-Ile-Ala), 91-101 (1,193 Da with the N-terminal sequence of Ile-Met-Val), and a modified 102-110 (1,055 Da with the N-terminal sequence of Gln-Tyr-Leu) fragments. The MS/MS of the modified fragment showed that it contains the 102-110 sequence of the wild type DHFR. However, modification with loss of 18 Da occurred at the C-terminal, involving Lys109 and Leu110, resembling that observed above in dissection at the 77th position. In this case, in contrast to the case at the 77th position, the amount of the modified fragment (fragment 4 in Table II) is only half of that of the normal 12,121 Da fragment (fragment 2 in Table II), and the major product is the dehydroalanine derivative (fragment 1 in Table II) resulting from the β-elimination reaction (route 2 in Fig. 1) of the cyanocysteine side chain.

CD Spectra of the Isolated Peptide Fragments—Using the isolated dissected fragments, DHFR activity and far UV CD spectra were measured. Figure 6 shows the CD spectra of the isolated fragments at low (15°C) and high (80°C) temperature. Although the CD spectrum of the 1-110 peptide was completely different from that of wild-

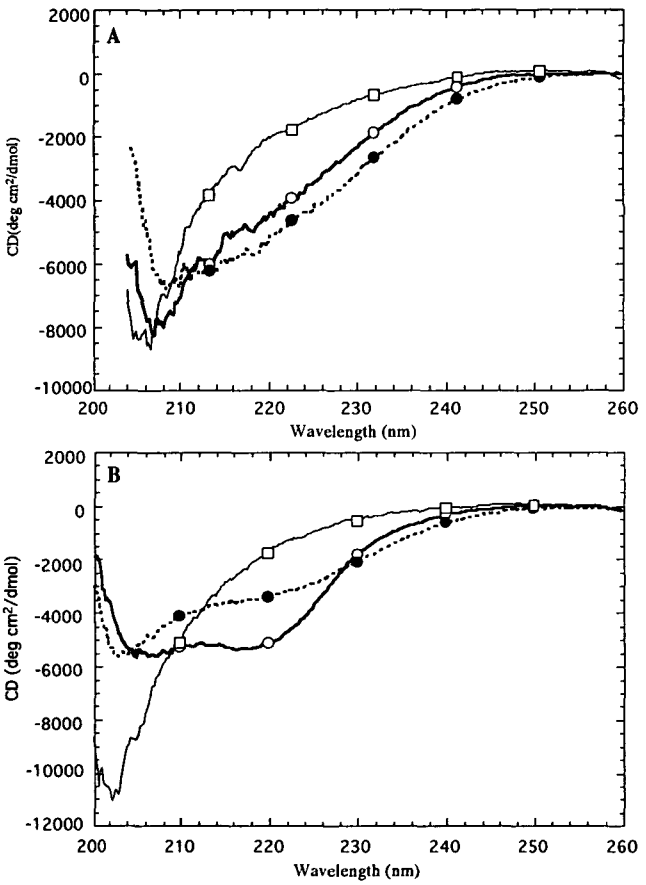

Fig. 6. **CD spectra of isolated peptides at low and high temperatures.** (A) peptides resulting from dissection at the 76-77 linkage; (○), the 1-76′ peptide (at 15°C); (●), the 1-76′ peptide (at 80°C); (□), the ITC-77-159 peptide. (B) peptides resulting from dissection at the 110-111 linkage; (○), the 1-110 peptide (at 15°C); (●), the 1-110 peptide (at 80°C); (□), the ITC-111-159 peptide.

TABLE II. **Summary of molecular mass measurements and N-terminal sequences of Tyr111→Cys DHFR and its derivatives.**

Protein or peptide	Molecular mass (Da)		N-terminal sequence		Assignment
	Measured	Calculated	From DNA Seq.	Experimental	
S-Cyanocysteine mediated dissection					
Protein 1	17,890	17,892	MISILAALAV	MISILAALAV	Tyr111→Cys DHFR
Modified protein	17,915	17,916	MISILAALAV	MISILAALAV	Cyanocysteine111 DHFR
Fragment 1	17,857	17,851	MISILAALAV	MISILAALAV	Dehydroalanine111 DHFR
Fragment 2	12,120	12,121	MISILAALAV	MISILAALAV	1-110 fragment
Fragment 3	5,812	5,812	CLTHIDAEVE	Not detected	111-159 fragment with ITC N-terminal
Fragment 4	12,103	12,121-18	MISILAALAV	MISILAALAV	1-110 fragment −18 Da (1-110′ fragment)
V8-protease digestion of the fragment 4 (1-110′ fragment)					
Fragment 5	5,489	5,489	MISILAALAV	MISIL	1-48 fragment
Fragment 6	3,520	3,520	SIGRPLPGRL	SIGRP	49-80 fragment
Fragment 7	913	913	AIAAAGDVPG	AIA	81-90 fragment
Fragment 8	1,193	1,193	IMVIGGGRVY	IMV	91-101 fragment
Fragment 9	1,055	1,073-18	QFLPKAQKLY	QFL	102-110 fragment −18 Da (102-110′ fragment)

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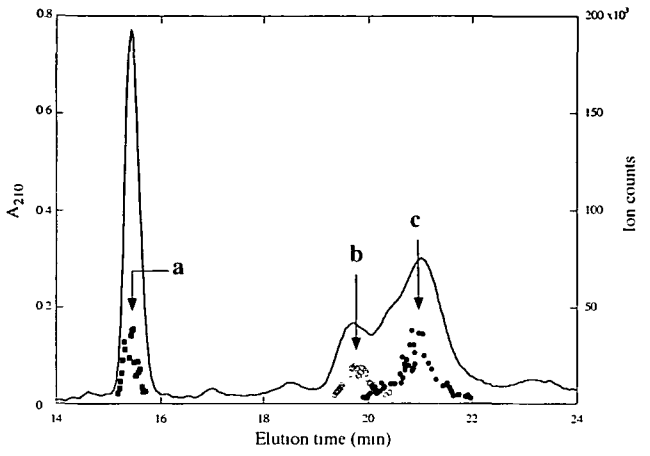

Fig. 7. **On-line LC/ESI-MS chromatogram of the reaction product of cyanocysteine77-DHFR with glycine.** (–), chromatogram detected in terms of absorbance at 210 nm; (■), ion counts of *m/z* value between 1,049 and 1,050, which is specific for the ITC-77-159 peptide; (○), ion counts of *m/z* value between 958 and 959, which is specific for the 1-76+Gly peptide; (●) ion counts of *m/z* between 949 and 950, which is specific for the 1-76′ peptide. The molecular masses of major compounds in peaks **a**, **b**, and **c** were 9,444, 8,623, and 8,548 Da, which correspond to the 77-159 fragment with the ITC-77-159 peptide, the 1-76+Gly peptide, and the 1-76′ fragment, respectively.

type DHFR, the double minimum shape of the spectrum suggested the presence of stable folding structure. It changed to the CD spectrum of a random coil at high temperature (80°C) where the wild-type DHFR is completely heat-denatured. Three other dissected fragments, namely, the 1-76′, ITC-77-159, and ITC-111-159 peptides, did not appear to contain stable folding structures. Although the 1-110 peptide contains all the amino acid residues, except Thr123, which participate in binding with the substrate (dihydrofolate) and coenzyme (NADPH), it did not show DHFR activity.

An attempt to direct expression of the DHFR coding region corresponding to the 1-110 fragment did not generate any active DHFR phenotype or result in accumulation of stable gene products in transformed *E. coli* cells. The mixing of the 1-110 and ITC-111-159 peptides led to precipitation at neutral pH and the DHFR enzyme activity could not be recovered. The failure of reconstitution of the activity with the two dissected fragments did not seem to be due to the ITC modification of the 111-159 peptide because an attempt at *in vivo* reconstruction of the 1-110 and 111-159 parts of DHFR by coexpression of the two fragments did not generate an active DHFR phenotype or result in any accumulation of stable gene products in transformed *E. coli* cells. Thus, the chain connectivity seems to be an important factor in retaining DHFR activity.

Gly-Mediated Dissection Reaction at Lys76-Cys77 Position and Lys109-Leu110-Cys111 Position—As discussed later, intramolecular nucleophilic attack on the carbonyl carbon at the X-cyanocysteine linkage by the ε-amino group of a lysine residue seems to take place in the dissection reaction of DHFR at the Lys76-Cys77 and Leu110-Cys111 positions. To examine whether this nucleophilic attack takes place in an intermolecular manner or not, the α-amino group of glycine was used as the nucleophile and the dissection reaction was carried out in the presence of 1 M glycine. As shown in Fig. 7, formation of 1-76+Gly peptide was observed in addition to the 1-76 fragment with the modified C-terminal upon dissection reaction at the Lys76-Cys77 position. Similarly, the formation of the 1-110+Gly fragment was observed in the dissection reaction at Leu110-Cys111 (data not shown).

Reaction Mechanism—We propose a novel reaction route (route 3 shown in Fig. 1) based on the reaction mechanism (route 1 and route 2) previously proposed by Jacobson *et al.* (*3*). The Lys-cyanocysteine linkage formed by modification of Ser77→Cys DHFR with NTCB would be converted to ε-caprolactam and ITC derivatives by intramolecular nucleophilic attack (route 3) of the ε-amino group. This intramolecular reaction would compete with intermolecular attack by hydroxide ion as a nucleophile (route 1) or as a base causing β-elimination (route 2). In the case of Tyr111→Cys DHFR (Lys109-Leu110-Cys111), intramolecular attack of the ε-amino group of Lys109 may also take place, resulting in a modified peptide with a molecular mass of 18 Da less than the expected value (route 3). However, the distance between the ε-amino group and the carbonyl group, which is related to the number of amino acids between Lys and cyanocysteine ["*n* (*n*=0, 1, 2, ...)" in Fig. 1], is large so that the efficiency of this nucleophilic attack is less than in the case of Ser77→Cys DHFR (Lys76-cyanocysteine77 linkage). Thus, the competitive route 1 and route 2 reactions are the major ones in the case of the Lys109-Leu110-cyanocysteine111 linkage. The α-amino group of glycine could attack the carbonyl carbon as a nucleophile, in place of hydroxide ion. Our preliminary studies using various nucleophiles revealed that at least primary amines can attack the carbonyl carbon (data not shown). This novel reaction is shown as route 4 in Fig. 1.

DISCUSSION

Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) (*27*) is a valuable technique for detailed structural characterization of fragment peptides and offers a more accurate and rapid approach than conventional gel-electrophoresis analyses. Sequencing techniques based on Edman degradation are of limited value for characterization of structurally modified amino acid residues, whereas tandem MS (MS/MS) (*28*) can readily accommodate such cases. Capillary electrophoresis (CE) is an alternative approach to characterize peptides and proteins. Since the separation is based on the charge and the size of solutes, different information, such as pK_a and the hydrodynamic radius, can be obtained.

In this paper, we have analyzed the reaction products of the cyanocysteine-mediated dissection reaction by LC/MS, LC/MS/MS, CE, and protein sequencing, and identified a modification reaction. The dissection reaction at the X-cyanocysteine linkage has been extensively studied by Catsimpoolas and Wood (*2*) and Jacobson *et al.* (*3*), and it has been shown that hydroxide ion works as a nucleophile to attack the carbonyl carbon at the linkage (route 1, Fig. 1). β-Elimination (route 2) also occurred as a side reaction caused by hydroxide ion working as a base (*2, 3*). We have demonstrated here that the carbonyl carbon at the X-cyanocysteine linkage is attacked by primary amine, such as the ε-amino group of lysine and the α-amino group of glycine.

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This nucleophilic attack takes place in an intramolecular or intermolecular manner, forming a new peptide bond (namely, an "*S*-cyanocysteine-mediated carbonyl-carbon activation" mechanism). A similar reaction has been reported for *S*-cyanoglutathione by Catsimpoolas and Wood (*2*) and Jacobson *et al.* (*3*). They found that the product of the cleavage reaction of *S*-cyanoglutathione was 2-pyrrolidone-5-carboxylic acid and suggested that the N-terminal α-amino group of *S*-cyanoglutathione might work as a nucleophile in the intramolecular reaction instead of hydroxide ion. These intramolecular reactions at the N-terminal or C-terminal of dissected products mean that care is needed in the characterization of the fragmented products. The *S*-cyanocysteine-mediated carbonyl-carbon activation mechanism provides a novel approach to non-catalytic peptide bond formation which should be useful for site-specific immobilization of proteins at the C-terminal and for segment condensation of peptides. Such studies have been successfully performed in our laboratory and the results will be reported elsewhere.

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