J. Biochem. 2009;145(4)517–523
doi:10.1093/jb/mvp003

Protein Oxidation During Long Storage: Identification of the Oxidation Sites in Dihydrofolate Reductase from *Escherichia coli* through LC–MS and Fragment Studies

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Received November 14, 2008; accepted January 7, 2009; published online January 16, 2009

An LC–MS study revealed some heterogeneity in terms of molecular mass of a cysteine-free mutant of dihydrofolate reductase (DHFR) after long storage of the highly purified protein as an ammonium sulfate precipitate, but not in the case of a cysteine- and methioneine-free mutant of DHFR. One-third of the cysteine-free DHFR sample stored for a long time, around 18 months, comprised molecular species with molecular masses increased by 16, 32 and 48 Da. A peptide mapping study revealed that at least one of the methionine residues at positions 1, 16 and 20 was oxidatively modified to a methione-sulfoxide residue, while those at positions 42 and 92 were essentially unaffected. Each of the oxidized species of the DHFR exhibiting different degrees or sites of oxidation was further purified to essentially homogeneity in terms of molecular mass from the stored sample, and its enzyme activity was determined. One oxidized DHFR showed higher activity than that of the non-oxidized enzyme, while the other four oxidized DHFRs showed less activity. This agrees with the observation that the enzyme activity of the stored sample, a mixture in terms of oxidation, was apparently the same as that of the non-oxidized enzyme. This suggests that the activity itself is not a proper measure for quality control of proteins.

Key words: dihydrofolate reductase, fragment studies, liquid chromatography-mass spectrometry (LC–MS), protein aging, protein oxidation.

Abbreviations: AS-DHFR, C85A/C152S variant of DHFR; DHF, dihydrofolate; DHFR, dihydrofolate reductase; k_{cat}, maximum velocity; K_m, Michaelis constant; LC–MS, liquid chromatography–mass spectrometry.

INTRODUCTION

Protein oxidation under oxidative stress *in vivo* is a recent subject as to understanding of the molecular mechanisms relevant to Alzheimer's disease (*1–5*). The oxidation of a single-methionine residue of amyloid β-peptide plays an important role in the expression of neurotoxicological properties (*2–5*). Age-dependent oxidation of methionine residues or accumulation of oxidatively modified proteins may be a measure of biological aging (*6–8*).

Also, *in vitro* oxidation of highly purified proteins and its consequence in terms of quality control of protein products should become more important subjects with the rapidly growing number of therapeutic proteins, such as monoclonal antibodies. In this context, the recent development of mass spectrometry has allowed us to detect heterogeneity of highly purified proteins at the molecular level (*9*, *10*). However, until now, few studies have been carried out on the characterization of an aged (stored) protein *in vitro* using well-assigned proteins in terms of the degree and site of oxidation in the protein.

Dihydrofolate reductase (DHFR) (EC 1.5.1.3) is a monomeric, two-domain protein that catalyses the reduction of dihydrofolate to tetrahydrofolate, using the reducing cofactor NADPH (*11*). DHFR is a clinically important enzyme not only as the target of a number of antifolate drugs such as trimethoprim and methotrexate, but also as the enzyme that produces *l*-leucovorin, an anti-cancer drug, in a sterospecific manner (*12*). Because overproduction of DHFR in its cells makes *Escherichia coli* trimethoprim-resistant (tmp^R), DHFR has been used not only as a selectable genetic marker, tmp^R (*13*), but also as a handle protein (affinity handle) for the production of peptides (*14*).

In previous studies (*15*, *16*), we found a cysteine-free (C85A/C152S) DHFR mutant, namely, AS-DHFR, which was successfully developed to avoid the molecular heterogeneity due to intra- or inter-molecular formation of S–S bond(s) and to overcome the reversibility against heat denaturation. Here, we report that great heterogeneity in molecular mass resulted in long storage of the DHFR from *E. coli*. The molecular heterogeneity and its functional consequence were studied to determine the degree of oxidation and the sites of oxidized amino-acid

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residues in the stored DHFR sample, by liquid chromatography mass spectrometry (LC–MS) and peptide mapping analyses.

MATERIALS AND METHODS

Protein Purification—DHFRs were purified on MTX (methotrexate)-agarose affinity (Sigma) and DEAE-Toyopearl 650M anion-exchange (Tosoh, Tokyo, Japan) columns as described previously (*14*). The purified DHFRs were stored as ammonium sulfate precipitates in 10 mM potassium phosphate (pH 6.8) buffer containing 0.2 mM EDTA and saturated with ammonium sulfate at 4°C. All proteins were dialysed against 10 mM potassium phosphate (pH 6.8) buffer containing 0.2 mM EDTA and 1 mM DTT before use.

LC–MS Measurements—Mass-spectrometric (MS) measurements were carried out in the positive-ion mode using a PE Sciex API III triple quadrupole mass spectrometer as described previously (*17*). Online LC–MS measurements were carried out as previously (*17*). Separation of the DHFRs was carried out with an acetonitrile gradient from 36% (0 min) to 42% (60 min) containing 0.1% TFA for L-column ODS (ϕ 4.6 × 150 mm; Kagaku-hin Kensa Kyoukai, Tokyo) at the flow rate of 1.0 ml/min. Separation of peptide fragments was performed with an acetonitrile gradient from 0% (0 min) to 60% (40 min) containing 0.1% TFA for L-column ODS (ϕ 2.1 × 150 mm) at the flow rate of 0.2 ml/min.

Enzyme Assay—DHFR activity was determined spectrophotometrically at 15°C by following the disappearance of NADPH and DHF at 340 nm (ε_{340} = 11,800 $M^{-1}cm^{-1}$) (*18*). The standard assay mixture comprised 50 μM DHF, 100 μM NADPH, 14 mM 2-mercaptoethanol, MTEN buffer (50 mM 2-morpholinoethanesulfonic acid, 25 mM Tris (hydroxymethyl) aminomethane, 25 mM ethanolamine, 100 mM NaCl, pH 7.0) and the enzyme. The reaction was initiated by adding DHF or the enzyme. Michaelis parameters (k_{cat} and K_m) were determined from the measurements with various concentrations of DHF (*19*).

Protease Digestion for Peptide Mapping—DHFR derivatives were separated from the stored sample by liquid chromatography with L-column ODS as described in the section on LC–MS measurements and each DHFR derivative was purified. The purified DHFR derivatives were digested with lysyl endopeptidase (Wako Pure Chemical Industries, Tokyo) in 20 mM Tris–HCl, pH 9.0, 1 mM EDTA and 1.2 M guanidine at 37°C overnight. The purified 1–32 peptide (the peptide fragment comprising residues 1 and 32) was digested with trypsin (Funakoshi, Tokyo, Japan) in 10 mM Tris–HCl (pH 7.5), 20 mM $CaCl_2$ and 0.6 M guanidine at 37°C for 4 h, and then digested with V8 protease (Wako Pure Chemical Industries, Tokyo) in 10 mM ammonium acetate, pH 4.0 and 0.6 M guanidine at 37°C overnight.

Oxidation with Hydrogen Peroxide—Equal volumes of various concentrations (0 μM to 200 mM) of hydrogen peroxide (H_2O_2) and 200 μM of the DHFR sample were mixed and stood for 12 h at room temperature. All samples were in 10 mM potassium phosphate (pH 6.8) buffer containing 0.2 mM EDTA. After the oxidation treatment, H_2O_2 was removed by dialysis.

Calculation of the Accessible Surface Area—The MOLMOL program (*20*) with the probe radius of the solvent set to 1.4 Å was used to obtain the accessible surface area value for each methionine residue and its sulfur atom in various X-ray crystal structures of DHFR listed in a previous paper (*21*). The structure coordinates used here were obtained by deleting substrates (methotrexate, folate and so on) and/or cofactors ($NADP^+$, NADPH and so on) from originally deposited structure coordinates for binary and/or ternary complexes.

RESULTS AND DISCUSSION

Analysis of Stored AS-DHFR—The AS-DHFR sample stored for almost 18 months at 4°C as an ammonium sulfate precipitate gave multiple protein peaks when analysed by LC–MS (Fig. 1). Six protein fractions exhibiting different molecular masses derived from the pure AS-DHFR were detected by total ion count profile (A to F in Fig. 1 and Table 1), although the peaks, D, E and F were not so clear. The LC–MS profile of the stored protein sample was different from that of a freshly purified sample giving a single-protein peak corresponding to a molecular mass of 17,953 Da. Each of protein fractions A to F in Fig. 1 was separated and pooled by repeated sampling after reversed phase HPLC similar to the LC–MS. The respective pooled fractions were further purified to give a single-protein peak exhibiting essentially homogeneity in molecular mass by the reversed phase HPLC, respectively.

The molecular mass of the protein in the fraction A was the same as that of the AS-DHFR predicted from its amino-acid sequences (17,953 Da). In contrast, those in the fractions B and C were 16 Da larger in molecular mass than the predicted values, and those in the fractions D, E and F were 32, 32 and 48 Da larger, respectively. One-third of the stored protein sample comprised molecules with increased molecular masses (Table 1). For activity measurements, the purified and pooled fractions were dialysed against 10 mM potassium phosphate

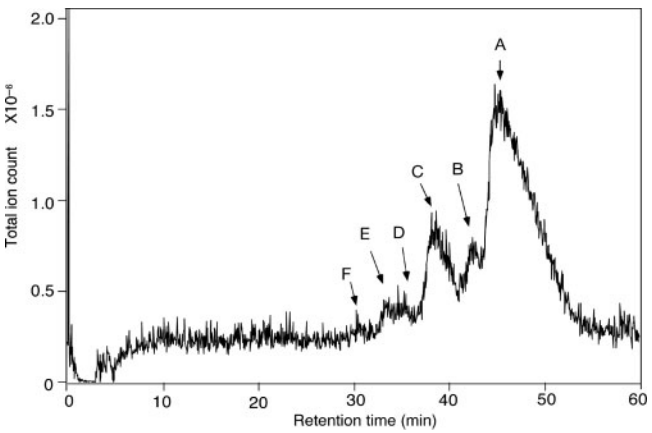

Fig. 1. **LC–MS chromatogram of stored AS-DHFR.** The profile of total ion counts with the detector of the mass spectrometer is shown. The detected peaks were designated as protein fractions A, B, C, D, E and F, respectively.

Table 1. **Summary of the molecular characteristics of protein fractions A, B, C, D, E and F detected for AS-DHFR after long storage.**

Protein fraction[a]	Molecular mass (Da)[b]	Amount (%)[c]	DHFR activity		
			Relative activity (%)[d]	k_{cat} (s^{-1})	K_m (μM)
A	17,953	68	100	4.7	1.5
B	17,969	8	64	ND[e]	ND
C	17,969	17	121	5.7	1.9
D	17,985	3	55	ND	ND
E	17,985	3	64	2.4	2.0
F	18,001	1	76	3.6	1.4
AS-DHFR[f]	17,953		100	4.9	1.2

[a]A, B, C, D, E and F correspond to the respective peaks detected for stored AS-DHFR on the LC–MS measurement shown in Fig. 1. [b]Molecular masses of the proteins in fractions A, B, C, D, E and F were determined by LC–MS. An error value for each estimated molecular weight (caused by an instrumental error) is ±3 Da. [c]Relative amounts of fractions A, B, C, D, E and F found for the sample were calculated from the total ion counts of individual molecules in Fig. 1. [d]Relative activity of each fraction when the activity of fraction A was set as 100%. [e]Not determined. [f]The data for freshly prepared AS-DHFR were essentially the same as those obtained in previous work by Iwakura *et al.* (*15*, *36*).

(pH 6.8) buffer containing 0.2 mM EDTA and 1 mM DTT. The resultant activity parameters are summarized in Table 1. The protein in fraction A showed very similar DHFR activity as well as k_{cat} and K_m values to the freshly purified AS-DHFR, while these values for the proteins in fractions B to F were different (Table 1). It is noteworthy that, although the proteins in fractions B to F showed different activities from each other, the aged AS-DHFR sample itself showed the same activity as the freshly prepared AS-DHFR (data not shown). Until this study, we had not been able to detect the molecular heterogeneity in stored protein samples by checking the enzymatic activity only. Why the relative activity could not be detected can be understood through the following calculation using the data in Table 1: (amount/100) × (relative activity) was calculated to be 98.02 [=0.68 × 100 (A) + 0.08 × 64 (B) + 0.17 × 121 (C) + 0.03 × 55 (D) + 0.03 × 64 (E) + 0.01 × 76 (F)], this value being accidentally very similar to the activity of the freshly prepared AS-DHFR. This strongly suggests that activity measurement of a whole mixed sample is not sufficient to detect the aging effect on a protein catalyst.

Identification of Oxidized Amino-Acid Residue(s) Related to Increases in Molecular Mass—To identify the oxidized amino-acid residue(s) in the AS-DHFR, six protein fractions, A to F were analysed by peptide mapping. Digestion of each protein with lysyl endopeptidase created six peptide fragments (Fig. 2). The molecular weights of five of the peptide fragments were determined under our LC–MS experimental conditions (Fig. 3). No molecular weight alteration was detected in the five peptide fragments from fraction A. In contrast, an increased molecular weight of the peptide fragment comprising positions 1 and 32 (1–32 peptide) was detected in fractions B, C, D, E and F (indicated by arrows in Fig. 3) and summarized in Table 2. For the 1–32 peptide from fractions B and C, an increase of 16 Da

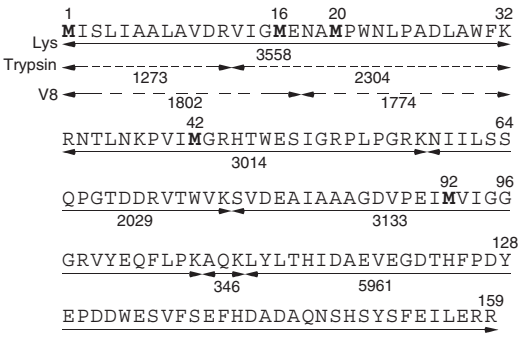

Fig. 2. **Amino-acid sequence of AS-DHFR and digested peptide fragments mapping.** The six peptide fragments derived from AS-DHFR digested with lysyl endopeptidase (denoted as 'Lys') are shown as solid arrows under the sequences. The peptide fragments derived from peptide 1–32 digested with trypsin or V8 protease are shown as dashed arrows. The numbers above the sequence and below the arrows indicate the site numbers and molecular masses of the corresponding peptides, respectively.

in molecular weight was found. For the 1–32 peptide from fractions D and E, an increase of 32 Da, and for that from fraction F, an increase of 48 Da, were also detected, respectively. These results proved that the molecular heterogeneity only involved the amino acids at positions 1–32, other sites being essentially unaffected.

It is well known that a methionine residue is oxidation-susceptible due to a highly reactive sulfur atom in its side chain (*22–25*). Also, it is known that the methionine sulfoxide residue, 16 Da larger than a methionine residue, is a major and stable product of air oxidation (*24–28*). Because the 1–32 peptide fragment contains three methionine residues (Fig. 2), air oxidation of methionine(s) located in this region possibly occurs with a resulting increase in molecular mass of AS-DHFR during storage. To examine this possibility, the respective 1–32 peptides from fractions A to F were further analysed by peptide mapping using trypsin or V8 protease (Table 2). In fraction B, the molecular mass of the 1–17 peptide containing Met-1 and Met-16 was 16 Da larger than calculated, while the molecular mass of the 1–12 peptide containing only Met-1 was unaffected. In fraction C, the two peptides (the 13–32 and 18–32 peptides) containing Met-20 were almost 16 Da larger, but the 1–17 peptide containing (Met-1 and) Met-16 showed no change. These results indicate that Met-16 in fraction B and Met-20 in fraction C are oxidized, respectively. In the same way, we found that in fractions D and E, two methionine residues, Met-16 and Met-20 were oxidized, and in fraction F, three methionine residues, Met-1, Met-16 and Met-20, were oxidized (Table 2).

Our peptide mapping analysis involving LC–MS also showed that each methionine oxidation took place at positions 1, 16 or 20 with a 16 Da increase in molecular mass, not a 32 or 48 Da one. This finding strongly indicates that the oxidation product is the methionine sulfoxide residue, and rules out the possibility that the oxidation product is a further oxidized form of a methionine residue, such as a methionine sulfone residue in which a sulfur atom is bound to two oxygen atoms and

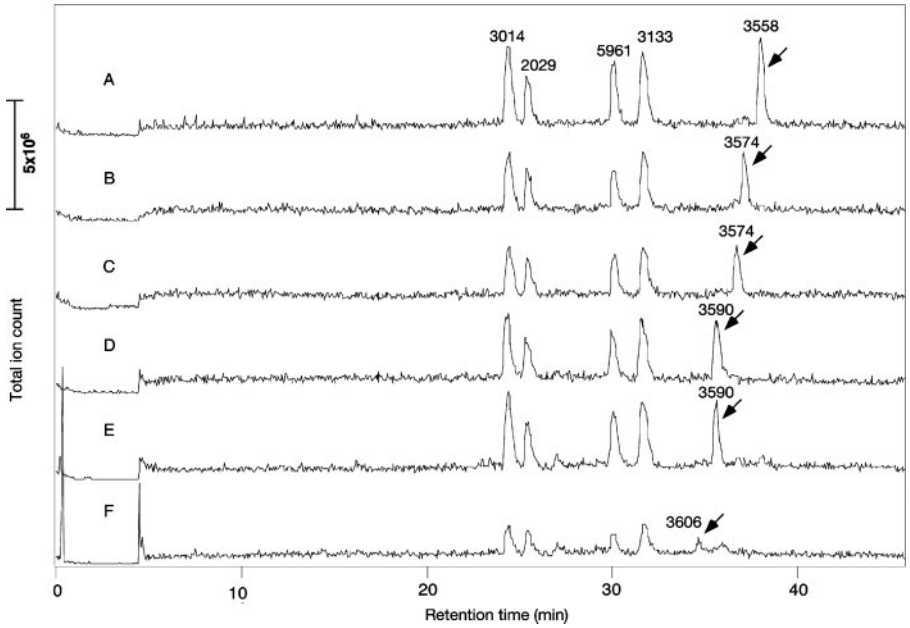

Fig. 3. **Analysis of the peptide fragments derived from AS-DHFR derivatives.** Purified fractions A, B, C, D, E and F were digested with lysyl endopeptidase and then the digested fragments were analysed by LC–MS. The profile of total ion counts with the detector of the mass spectrometer is shown. Each number indicates the molecular weight of the respective detected peak of the peptide fragment determined by MS analysis. Peaks with arrows correspond to fragments of peptide 1–32. An error value for each estimated molecular weight (caused by an instrumental error) is ±3 Da.

Table 2. **Molecular weights of peptide 1–32 derived from DHFR derivatives and of its fragments on further digestion.**

Protein fraction[a]	Molecular mass (Da)[b]					Modified residues[c]
		Trypsin digestion		V8 protease digestion		
	Peptide 1–32	Peptide 1–12	Peptide 13–32	Peptide 1–17	Peptide 18–32	
A	3,558 (±0)	1,273 (±0)	2,303 (−1)	1,802 (±0)	1,774 (±0)	None
B	3,574 (+16)	1,273 (±0)	ND[d]	1,818 (+16)	1,774 (±0)	Met-16
C	3,574 (+16)	1,273 (±0)	2,319 (+15)	1,802 (±0)	1,790 (+16)	Met-20
D	3,590 (+32)	1,273 (±0)	ND	1,818 (+16)	1,790 (+16)	Met-16, Met-20
E	3,590 (+32)	1,273 (±0)	2,335 (+31)	1,818 (+16)	1,7903 (+16)	Met-16, Met-20
F	3,606 (+48)	1,289 (+16)	2,335 (+31)	1,834 (+32)	1,790 (+16)	Met-1, Met-16, Met-20
(Calculated value)[e]	3,558	1,273	2,304	1,802	1,774	

[a]A, B, C, D, E and F correspond to the respective peaks detected for stored AS-DHFR on the LC–MS measurement shown in Fig. 1. [b]Molecular weights of peptide 1–32 from fractions A, B, C, D, E and F, and of its fragments on further digestion with trypsin and V8 protease were determined by LC–MS. The values in parentheses are differences in molecular weights from the respective calculated values. An error value for each estimated molecular weight (caused by an instrumental error) is ±3 Da. [c]Modified residues were deduced from the results of peptide mapping analysis. [d]Not determined. [e]Molecular weight of each peptide calculated from its primary sequence.

which shows a 32 Da increase in molecular weight compared to in the case of a methionine residue (*29*, *30*).

It is noteworthy that all the methionine residues (Met-42 and Met-92) in AS-DHFR other than Met-1, Met-16 and Met-20 were essentially unaffected by oxidation during the long storage, because a molecular mass change was only found for the 1–32 peptide.

The fractions D and E showed the same molecular mass and the same modified sites, suggesting that the two proteins in D and E fractions may be the same molecular species in terms of amino-acid sequence with a modified amino acid. At present, we do not have any data to suggest why the same protein species was distinctly separated with the HPLC. One possibility is that some further oxidation with small mass changes may take place. Other possibility is that two distinct conformers may exist and be separated by the HPLC.

Oxidation of AS-DHFR by Hydrogen Peroxide—It is known that methionine residues in proteins are readily oxidized by many oxidants, such as hydrogen peroxide (H_2O_2), hypochlorite, nitric oxide and superoxide (*22*, *27*, *29*). Among them, H_2O_2 is one of the most widely used reagents for methionine oxidation, specifically oxidizing methionine residues to methionine sulfoxide ones (*22*, *26*, *29*, *31*).

To further confirm the above conclusion regarding the molecular mass change during long storage, freshly purified AS-DHFR was treated with different concentrations of H_2O_2, and the resultant products were similarly analysed to the stored protein by LC–MS. Six fractions,

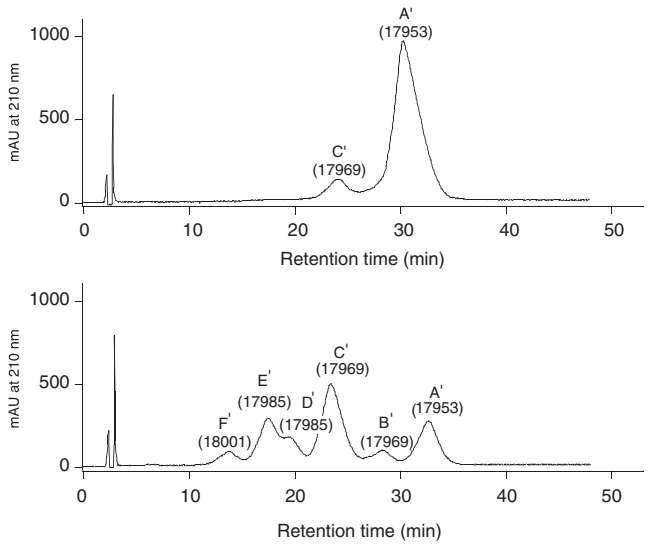

Fig. 4. **LC–MS chromatogram of AS-DHFR oxidized with H_2O_2.** AS-DHFR (100 μM) treated without (upper) and with (lower) 2 mM H_2O_2 was analysed by LC–MS. In the LC–MS chromatogram, six peaks, A′, B′, C′, D′, E′ and F′, are visible and the numbers indicate the molecular weights determined for the respective fractions. Molecular masses of the proteins in fractions A′, B′, C′, D′, E′ and F′ determined by LC–MS were 17,953 Da, 17,969 Da, 17,969 Da, 17,985 Da, 17,985 Da and 18,001 Da, respectively, and an error value for each estimated molecular weight (caused by an instrumental error) is ±3 Da.

namely A′, B′, C′, D′, E′ and F′, were obtained with molecular masses increased by 0, 16, 16, 32, 32 and 48 Da for the oxidized AS-DHFR treated with H_2O_2, respectively (Fig. 4). This is similar to the case of the aged AS-DHFR.

Fractions A′ to F′ seemed to vary as a function of the H_2O_2 concentration and the relative amounts were estimated from the LC–MS chromatograms (Table 3). As the H_2O_2 concentration became higher, the amount of fraction A′ simply decreased and that of fraction F′ simply increased, the amounts of fractions of C′ and E′ increasing to peaks (with 2 mM and 5 mM H_2O_2, respectively) and then decreasing gradually. With each H_2O_2 concentration, fractions B′ and D′ were minor products. The peptide mapping study involving LC–MS with lysyl-endopeptidase also showed that a molecular mass change was only found for the 1–32 peptide, similarly to the same analysis involving the aged AS-DHFR (data not shown).

It is noteworthy that fraction C′ was detected for the sample untreated with H_2O_2, although the amount was very small (Fig. 4). This may be because some air oxidation took place during incubation for 12 h at room temperature without a reducing agent (see MATERIALS AND METHODS). As indicated above, in fraction C, which may be equivalent to fraction C′, Met-20 was oxidized. This may indicate that Met-20 is the most sensitive to air oxidation. Also, methione oxidation may easily occur at room temperature without a reducing agent such as DTT.

Relationship between Structural Features and the Degree of Oxidization—DHFR from *E. coli* contains five

Table 3. **Relative amounts of protein fractions A′, B′, C′, D′, E′ and F′ derived by treatment with hydrogen peroxide.**

Concentration of H_2O_2	Relative amount (%)[a]					
	A′	B′	C′	D′	E′	F′
AS-DHFR						
Non	90	ND[b]	10	ND	ND	ND
10 μM	90	ND	10	ND	ND	ND
20 μM	90	ND	10	ND	ND	ND
50 μM	90	ND	10	ND	ND	ND
100 μM	88	ND	12	ND	ND	ND
200 μM	80	4	16	ND	ND	ND
500 μM	66	5	24	2	3	ND
1 mM	43	8	35	4	8	2
2 mM	20	7	38	10	19	6
5 mM	2	2	14	11	37	34
10 mM	ND	ND	2	4	23	71
20 mM	ND	ND	ND	ND	5	95
50 mM	ND	ND	ND	ND	ND	100
100 mM	ND	ND	ND	ND	ND	100
(ANLYF-DHFR)[c]						
Non	100	ND	ND	ND	ND	ND
10 mM	100	ND	ND	ND	ND	ND
100 mM	100	ND	ND	ND	ND	ND

[a]AS-DHFR (100 μM) was treated with various concentrations of hydrogen peroxide and then analysed by LC–MS (Fig. 4). The relative amounts of fractions A′ (17,953 Da), B′ (17,969 Da), C′ (17,969 Da), D′ (17,985 Da), E′ (17,985 Da) and F′ (18,001 Da), derived from the oxidized AS-DHFR, were determined from these respective peaks in LC–MS chromatograms (Fig. 4). [b]Not detected. [c]Cys- and Met-free mutant of DHFR, ANLYF-DHFR (M1A/M16N/M20L/M42Y/M92F/C85A/C152S) (100 μM), was also treated with 0 mM, 10 mM, and 100 mM hydrogen peroxide and then analysed by LC–MS (Data not shown). There was only one peak (corresponding to A′ of AS-DHFR) under the all conditions and the molecular weight of the protein in this peak is 17,908 ± 4 Da. Molecular weight of ANLYF-DHFR calculated from its primary sequence is 17,904 Da.

methionine residues at positions 1, 16, 20, 42 and 92, their oxidation susceptibilities differing from each other, as described above. Positions 1, 16 and 20 are located near the surface of the DHFR structure (*21*, *32*) (Fig. 5), and positions 42 and 92 are fully buried, as the accessible surface area (ASA) and temperature factor (B-factor) indicate in Supplementary Tables I and II.

When the oxidation reaction was carried out under a denaturing condition such as by using 6 M GuHCl, new protein peaks with increased molecular mass were observed, indicating that probably further oxidation took place at Met-42 and Met-92 (Supplementary Fig. 1). The chance to come into contact with the solvent may be a great factor for the oxidation.

Regarding Met-1, Met-16 and Met-20, of which the side chains seem to be fully exposed to the solvent, the degree of the oxidation in the aged sample seems to be Met-20 > Met-16 ≫ Met-1. This order is not always reflected to the ASA or the B-factor. Met-16 and Met-20, but not Met-1, are located in the mobile Met-20 loop of DHFR (*33*, *34*). The Met-20 loop is known to play an important role in DHFR activity due to its large and flexible loop movement and to be disordered in the apoenzyme and the $NADP^+$-holoenzyme crystal structures, an opening of

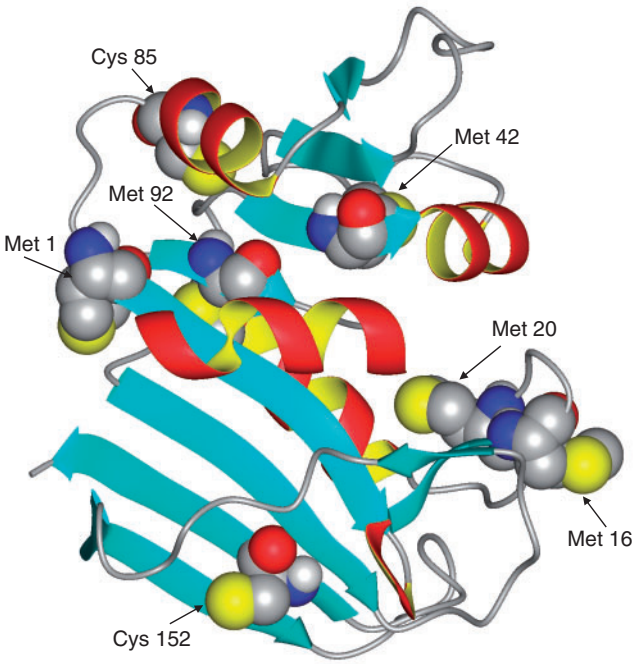

Fig. 5. **Location of methionine and cycteine residues in the DHFR structure.** Methionine and cysteine residues in the DHFR structure (PDB code: 1rx1) are shown as space-filling models. The DHFR structure was generated with the MOLMOL program (*20*).

the active-site cavity to the solvent being generated (*21*, *34*). A hydrogen exchange study on DHFR also indicated that amide protons in disordered regions, such as loops, are susceptible to deuterium exchange, those in Met-20 loop being especially completely and rapidly replaced by deuterium (*35*). It may be possible that main chain and side-chain motions in the protein accelerate the oxidation reaction, and this is the simplest explanation for why Met-20 is the most susceptible to oxidation. Taking into account these findings, we designed a highly active Cys- and Met-free DHFR with high resistance against oxidation (*36*).

Others—Although oxidatively modified proteins are known to be produced *in vivo*, these proteins are repaired by methionine sulfoxide reductase (*24*, *37–39*). This repair system acts as one of the systems for quality control of proteins *in vivo*. The use of the *in vivo* system would also help to restore the oxidized proteins *in vitro*.

As previously reported, we have developed Cys- and Met-free DHFRs, and showed that such proteins are indeed stable against oxidation when treated with high concentration of H_2O_2 (*36*, Table 3).

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

ACKNOWLEDGEMENT

We thank Ms Aiko Fujisawa, Ms Meiko Ohose and Ms Chiori Yamane, National Institute of Advanced Industrial Science and Technology, for the protein purification and enzyme assays.

CONFLICT OF INTEREST

None declared.

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