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In vitro methionine oxidation of *Escherichia coli*-derived human stem cell factor: Effects on the molecular structure, biological activity, and dimerization

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Abstract

The effect of oxidation of the methionine residues of *Escherichia coli*-derived recombinant human stem cell factor (huSCF) to methionine sulfoxide on the structure and activity of SCF was examined. Oxidation was performed using hydrogen peroxide under acidic conditions (pH 5.0). The kinetics of oxidation of the individual methionine residues was determined by quantitation of oxidized and unoxidized methionine-containing peptides, using RP-HPLC of Asp-N endoproteinase digests. The initial oxidation rates for Met^{159}, Met^{-1}, Met^{27}, Met^{36}, and Met^{48} were 0.11 min^{-1}, 0.098 min^{-1}, 0.033 min^{-1}, 0.0063 min^{-1}, and 0.00035 min^{-1}, respectively, when SCF was incubated in 0.5% H_2O_2 at room temperature. Although oxidation of these methionines does not affect the secondary structure of SCF, the oxidation of Met^{36} and Met^{48} affects the local structure as indicated by CD and fluorescence spectroscopy. The 295-nm Trp peak in the near-UV CD is decreased upon oxidation of Met^{36}, and lost completely following the oxidation of Met^{48}, indicating that the Trp^{44} environment is becoming significantly less rigid than it is in native SCF. Consistent with this result, the fluorescence spectra revealed that Trp^{44} becomes more solvent exposed as the methionines are oxidized, with the hydrophobicity of the Trp^{44} environment decreasing significantly. The oxidations of Met^{36} and Met^{48} decrease biological activity by 40% and 60%, respectively, while increasing the dissociation rate constant of SCF dimer by two- and threefold. These results imply that the oxidation of Met^{36} and Met^{48} affects SCF dimerization and tertiary structure, and decreases biological activity.

Keywords: dimerization; methionine oxidation; stem cell factor; structure/function

Stem cell factor acts on early hematopoietic progenitor cells (Martin et al., 1990; Zsebo et al., 1990) and also regulates the development and function of other cellular lineages that express its receptor c-kit, including mast cells, germ cells, and melanocytes (Zsebo et al., 1990; Galli et al., 1994). It works synergistically with other factors, such as IL-1, IL-3, IL-6, IL-7, G-CSF, GM-CSF, and erythropoietin (Anderson et al., 1990; Martin et al., 1990; Nocka et al., 1990; Zsebo et al., 1990; McNiece et al., 1991a, 1991b). The structural and biophysical characterization of CHO cell-derived and *Escherichia coli*-derived recombinant SCF has been reported (Arakawa et al., 1991). Both forms of SCF exist as noncovalently associated dimers and are rich in α-helical structure. We have demonstrated that the subunits of the SCF dimer exchange spontaneously under nondenaturing conditions. The dissociation rate constant of *E. coli*-derived SCF dimer was determined to be around 1.35×10^{-4} s^{-1} in sodium acetate buffer, pH 4.8, at 25 °C (Lu et al., 1995).

The oxidation of methionine to methionine sulfoxide during storage, sample handling, and in the aerobic biological environment where oxygen radicals are ubiquitous, has been known for decades (Manning et al., 1989). The nonenzymatic posttranslational modification occurs both in vivo and in vitro, and has been associated with the loss of biological activity in a wide variety of proteins and peptides (Coltrera et al., 1980; Vale et al., 1981; Amiconi et al., 1989; Manning et al., 1989). The oxidation of some of the methionine residues of growth hormone, which has been reported previously, reduced its affinity for lactogenic receptor and its in vitro biological potency (Teh et al., 1987). In this paper, we discuss the accessibility of the individual

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Abbreviations: SCF, stem cell factor; CHO, Chinese hamster ovary; RP-HPLC, reversed-phase HPLC; PBS, phosphate-buffered saline; IL, interleukin; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte and macrophage-colony stimulating factor; DTT, dithiothreitol; CD, circular dichroism.

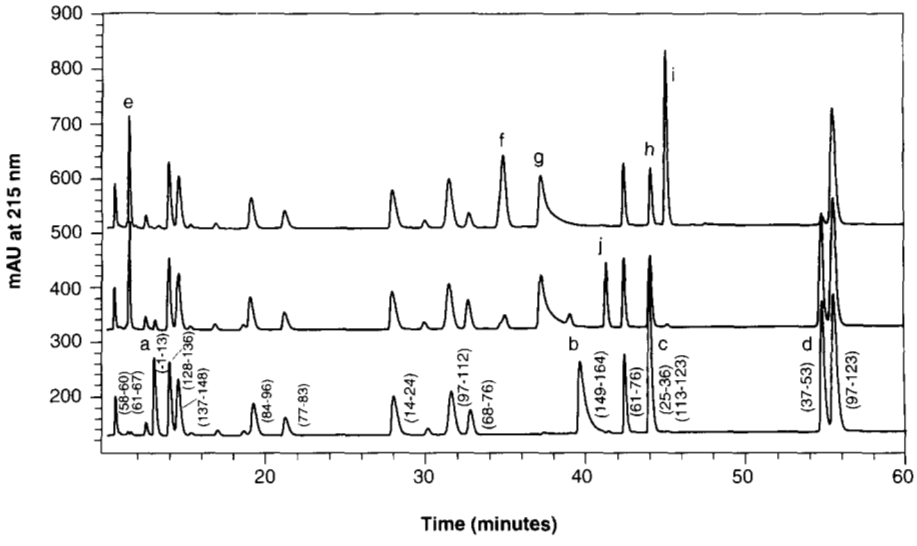

Fig. 1. A comparison of Asp-N peptide maps of methionine-oxidized SCF species to that of native SCF. Top: An Asp-N peptide map of a methionine-oxidized SCF sample, obtained by incubating SCF with 3% H_2O_2 for 26 h at room temperature. Middle: An Asp-N peptide map of a methionine-oxidized SCF sample, obtained by incubating SCF with 0.25% H_2O_2 for 12 h at 0 °C. Bottom: An Asp-N peptide map of native SCF. Numbers in parentheses indicate the assigned position in SCF sequence (Martin et al., 1990). Peptide (61–67) and peptide (68–76) resulted from nonspecific cleavage of Asp-N endoproteinase on the amino site of Glu^{68} of the peptide (61–76). For the same reason, peptide (97–112) and peptide (113–123) came from peptide (97–123). Peptide DAFK (54–57) and peptide DSLT (124–127) eluted much earlier in the nonreduced Asp-N peptide map (data not shown). The two peptides were coeluted with DTT peak in the reduced Asp-N peptide map.

methionine residues in *E. coli*-derived recombinant human SCF, Met^{-1}, Met^{27}, Met^{36}, Met^{48}, and Met^{159}, to oxidation by hydrogen peroxide, and the effects of the oxidation of these methionines on receptor binding, SCF-induced mitogenesis, protein conformation, and the dimerization dissociation rate constant.

Results

The oxidation of proteins by hydrogen peroxide under acidic conditions results in the conversion of methionine residues to methionine sulfoxide (Teh et al., 1987; Manning et al., 1989). In the peptide map of the oxidized protein, the addition of a single oxygen atom to methionine results in an increase in the polarity of the methionine sulfoxide-containing peptide. As a result, the oxidized peptide will elute at an earlier retention time on RP-HPLC. A comparison of an Asp-N endoproteinase peptide map of SCF with extensive H_2O_2 oxidation to that of unmodified SCF shows that the four peaks a, b, c, and d of unmodified SCF have shifted to earlier retention times, corresponding to peaks e, g, f, and i, respectively, of the oxidized protein (Fig. 1). Sequence analysis demonstrated that the sequences of the corresponding peaks are exactly the same. By mass spectrometry analysis, peaks e, g, and i all have 16 mass units more than the corresponding peaks, a, b, d; peak f has 32 mass units more than peak c (Table 1). Therefore, Met^{-1}, Met^{159}, Met^{48}

Table 1. *Laser desorption mass spectrometric analysis and sequence analysis of the methionine-containing peptides shown in Figure 1*

Peptide[a]	Retention time (min)	Sequencing data	Fragment	Theoretical MH^+ Average mass	Observed MH^+ Average mass
Peptide e	11.5	M-E-G-I-C-R-N-R-V-T-N-N-V-K	−1–13	1,634.9	1,651.0
Peptide a	13.1	M-E-G-I-C-R-N-R-V-T-N-N-V-K	−1–13	1,634.9	1,635.0
Peptide g	37.3	D-S-R-V-S-V-T-K-P-F-M-L-P-P-V-A-A	149–165	1,816.2	1,832.0
Peptide b	39.7	D-S-R-V-S-V-T-K-P-F-M-L-P-P-V-A-A	149–165	1,816.2	1,815.8
Peptide f	35	D-Y-M-I-T-L-K-Y-V-P-G-M	25–36	1,431.8	1,463.7
Peptide j	41.4	D-Y-M-I-T-L-K-Y-V-P-G-M	25–36	1,431.8	1,447.5
Peptide c	44.1	D-Y-M-I-T-L-K-Y-V-P-G-M	25–36	1,431.8	1,431.5
Peptide i	45.2	D-V-L-P-S-H-C-W-I-S-E-M-V-V-Q-L-S	37–53	1,944.3	1,960.7
Peptide d	54.9	D-V-L-P-S-H-C-W-I-S-E-M-V-V-Q-L-S	37–53	1,944.3	1,944.7
Peptide h	44.1	E-E-F-F-R-I-F-N-R-S-I	113–123	1,458.7	1,458.9

[a] Peptide h and peptide c coeluted in peak c. Peptide h is not a methionine-containing peptide.

in peptides e, g, i, respectively, and the two methionines at positions 27 and 36 in peptide f have been oxidized to form methionine sulfoxide.

No other peptides disappear or change retention time even following 26 h of oxidation in the presence of 3% hydrogen peroxide, pH 5.0, at room temperature. Amino acid sequence and mass spectrometric analysis of other Asp-N endoproteinase peptides demonstrated that all had the correct sequence assignment, sequencing recoveries were the same as for the peptides of unmodified SCF, and all had the expected peptide masses. In particular, recovery of Trp-containing peptide was good. Also, there is only one Trp in SCF, for which a gradual red shift of the fluorescence maximum and an increase in fluorescence intensity with increasing SCF oxidation were observed (see later in Fig. 6). This suggests that the majority of the Trp was not modified, because the fluorescence spectra of Trp between 300 nm and 400 nm would disappear upon oxidation (Guptasarma & Balasubramanian, 1992). These results indicate that amino acids of SCF, other than the methionine residues, remained unmodified during the oxidation experiments.

Peptide D-Y-M^{27}-I-T-L-K-Y-V-P-G-M^{36} eluted as peak c in the case of unmodified SCF. Oxidation shifted its elution position to earlier retention times, from c to j and further to f. Mass spectrometric analysis of peptide j showed an increase of only 16 mass units, indicating it contains only one oxidized methionine. In order to determine which methionine, Met^{27} or Met^{36}, is oxidized in peptide j, peptides c, j, and f were digested separately with trypsin and the resulting peptides were analyzed further. Two peptides, D-Y-M^{27}-I-T-L-K and Y-V-P-G-M^{36}, were obtained following the tryptic digestion of peptide c (Fig. 2). Peptide f yielded peptides that both contained oxidized methionine and eluted earlier, as expected. Peptide j also yielded two peptides. The first one, D-Y-M^{27}-I-T-L-K, eluted at the earlier retention time, indicating Met^{27} was oxidized. The other one, Y-V-P-G-M^{36}, eluted at the original retention time, indicating Met^{36} was not oxidized. Thus, it appears that Met^{27} is oxidized before Met^{36}.

The kinetics of oxidation of the individual methionine residues was determined by integrating the area of the methionine-containing peptide peaks following incubation with H_2O_2 for various times, using 0.5 mg/mL SCF. Hydrogen peroxide was removed immediately after completing the incubation by injecting the samples onto an anion exchange DEAE-5PW HPLC column; the preparations were then subjected to Asp-N endoproteinase digestion. The integrated areas of the methionine sulfoxide-containing peaks in the peptide maps at different time points were divided by those of the corresponding peaks in the SCF sample following the complete oxidation to obtain the percent oxidation for each methionine. To eliminate slight quantitative deviations due to sample handling and instrumentation, the integrated areas of all peaks were normalized to the integrated area of a reference peak, peak T28. The percent oxidation of the individual methionine residues was plotted as a function of reaction time (Fig. 3). Eighty-five percent of Met^{-1} and Met^{159} were oxidized within 15 min, whereas 40% of Met^{27}, 7% of Met^{36}, and <1% of Met^{48} were oxidized during the same period. The initial oxidation rate of each methionine residue was determined as the percent of oxidation versus reaction time, assuming first-order kinetics. The initial oxidation rates for Met^{159}, Met^{-1}, Met^{27}, Met^{36}, and Met^{48} are 0.11 min^{-1}, 0.098 min^{-1}, 0.033 min^{-1}, 0.0063 min^{-1}, and 0.00035 min^{-1}, respectively.

Met^{-1} and Met^{159} were oxidized very rapidly. This suggested that both these methionine residues are located on the external surface of the SCF molecule. Met^{27} oxidation occurred at an intermediate rate. This methionine resides in an environment less exposed to solvent. The micro environment of Met^{36} and Met^{48}, in which the oxidation rates are slow, results in less access for hydrogen peroxide, indicating that these residues are located in a more hydrophobic region.

To gain a greater understanding of the effects of methionine oxidation on the molecular structure and biological function of SCF, several different oxidation conditions were used to generate species that represent SCF with mainly two methionines (−1,

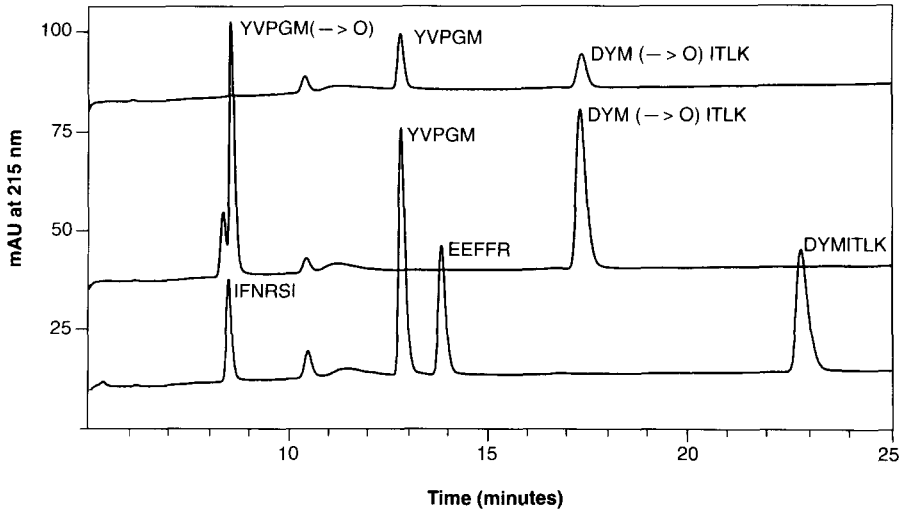

Fig. 2. HPLC chromatographies of tryptic digests of methionine-containing peptides c, j, and f. Bottom: RP-HPLC of trypsin digests of peptide c. Middle: Peptide f. Top: Peptide j. Peptide h (113–123), which coeluted with peptide c (25–36), is also cleaved into two peptides: peptide E^{113}-E-F-F-R and peptide I^{118}-F-N-R-S-I. M(→O) represents methionine sulfoxide.

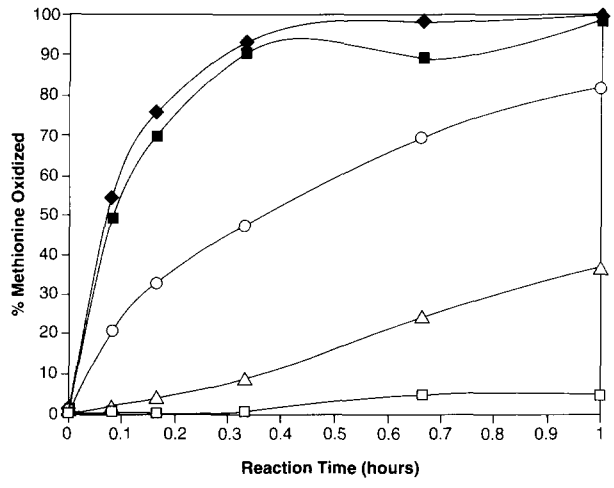

Fig. 3. Oxidation kinetics of the individual methionines in SCF plotted as the percentage of oxidation of individual methionine residues versus the incubation period. Met^{159} (◆); Met^{-1} (■); Met^{27} (○); Met^{36} (△); and Met^{48} (□). Standard deviations of recovery of these methionine-containing peptides were under 5% obtained from five repeating runs of the same sample on HPLC.

159) oxidized, three methionines (−1, 159, 27) oxidized, four methionines (−1,159, 27, 36) oxidized, and all five methionines (−1, 159, 27, 36, 48) oxidized. Formation and isolation of these methionine-oxidized species is described in the Materials and methods. These species were then analyzed by RP-HPLC (Fig. 4). As additional methionines are oxidized, elution occurs at increasingly earlier retention times. Each species is about 80% pure based on the RP-HPLC chromatographic profile and Asp-N peptide maps.

Conformation of oxidized SCFs

The far-UV CD spectra of the different oxidized SCF species and native SCF are shown in Figure 5B, and are identical within experimental error. The spectra are consistent with proteins containing substantial α-helix (about 50%) and indicate that methionine oxidation does not affect the secondary structure of SCF. As is often the case for helical proteins, the α-helical content is slightly enhanced at the decreased pH compared with that of native SCF in PBS.

The near-UV CD spectra of the oxidized SCF species and native unmodified SCF all contain a slight negative shift from the disulfides superimposed on the distinct fine structure attributable to the Phe (260–270 nm) and Tyr (270–285 nm). However, the 295-nm peak attributable to Trp is greatly reduced in the SCF species with four oxidized Met (159, −1, 27, 36), and has disappeared entirely from the spectra of the SCF species with all five Met (159, −1, 27, 36, 48) oxidized (Fig. 5A). This indicates that the oxidation of the Met^{36} and Met^{48} results in a loosening of the structure, such that Trp^{44} is in a much less asymmetric environment than that of the native protein.

A gradual red shift of the fluorescence maximum and an increase in fluorescence intensity with increasing Met oxidation were observed (Fig. 6). SCF contains only one Trp, located in a fairly hydrophobic environment, with a fluorescence maximum of 320 nm (Arakawa et al., 1991). The fluorescence maximum shifts from 320 nm in the native SCF sample to 330 nm in the species with all five methionines oxidized. The Trp environment becomes gradually more solvent exposed, and less quenched, as more Met are oxidized.

From the above analysis, it appears that oxidation of all of the methionines does not affect the secondary structure of the SCF, but that oxidation of Met^{36} and Met^{48} affects the local structure in the region around Trp^{44}. The near-UV CD spectra demonstrate that the 295-nm Trp peak is decreased following

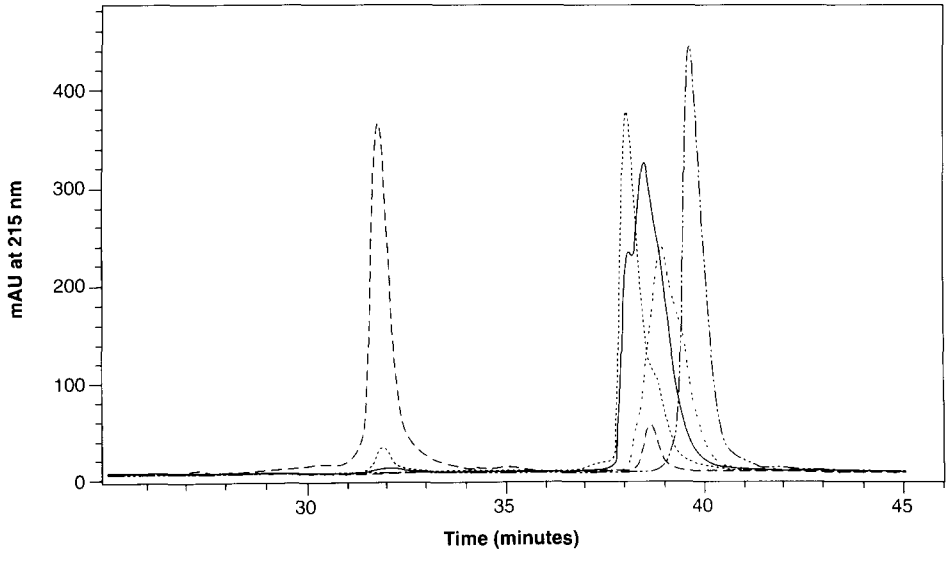

Fig. 4. RP-HPLC of SCF species with two, three, four, or five methionines oxidized. Native SCF (– – – – –); the two methionine-oxidized species (– – – –), generated by incubating SCF in 0.25% H_2O_2 for 4.5 h at 0 °C; the three methionine-oxidized species, (————), generated by incubating SCF in 0.25% H_2O_2 for 12 h at 0 °C; the four methionine oxidized species (– – – – –), generated by incubating SCF in 0.5% H_2O_2 for 3 h at room temperature; and the five methionine oxidized species (– – – –), generated by incubating SCF in 3% H_2O_2 for 26 h at room temperature.

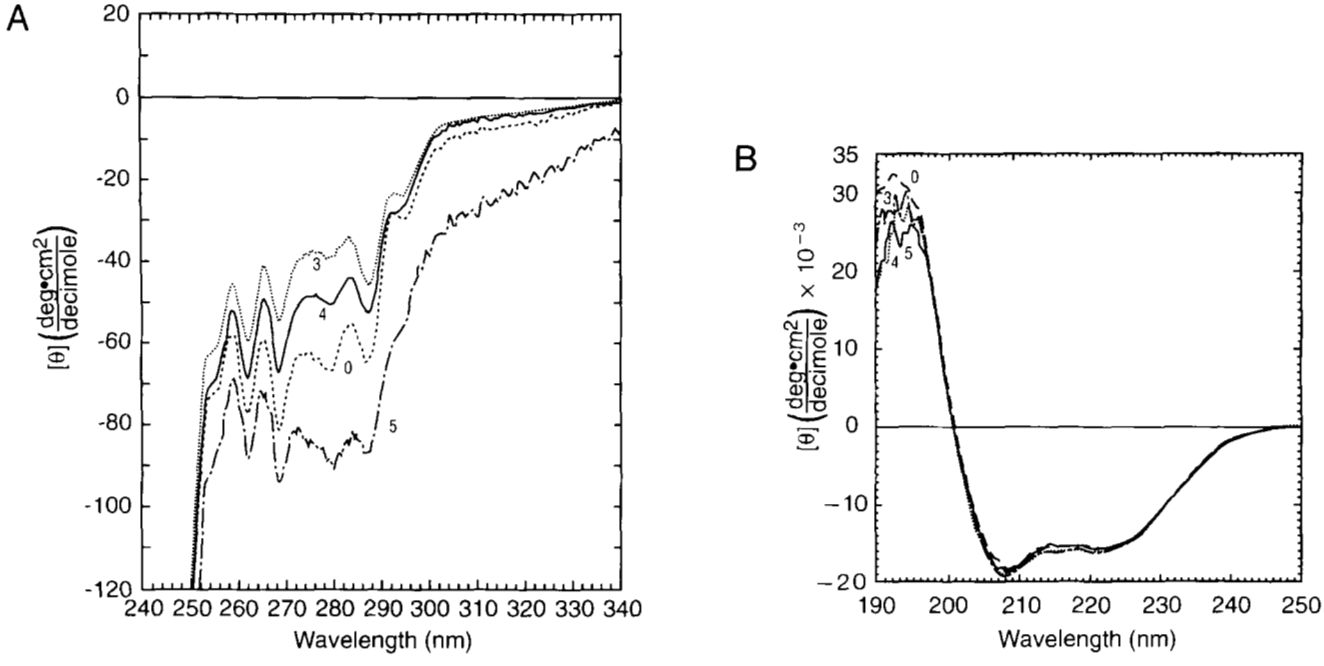

Fig. 5. CD spectra of methionine-oxidized SCF species. **A:** Near-UV spectra of the native SCF (0), the five methionine-oxidized species (5), the three methionine-oxidized species (3), and the four methionine-oxidized species (4). **B:** Native SCF and the three, four, and five methionine-oxidized species possess identical far-UV CD spectra. The difference in intensity seen between the SCF with three and four methionines oxidized and that of the native and the five oxidized methionine species results from the very low protein concentration of the later two samples coupled with the weak near-UV CD signal of SCF. The signal to noise ratio is different for these samples. This difference is enough to affect the intensity, but not shape, of the spectra. Similar results were obtained upon lowering the protein concentration of native SCF.

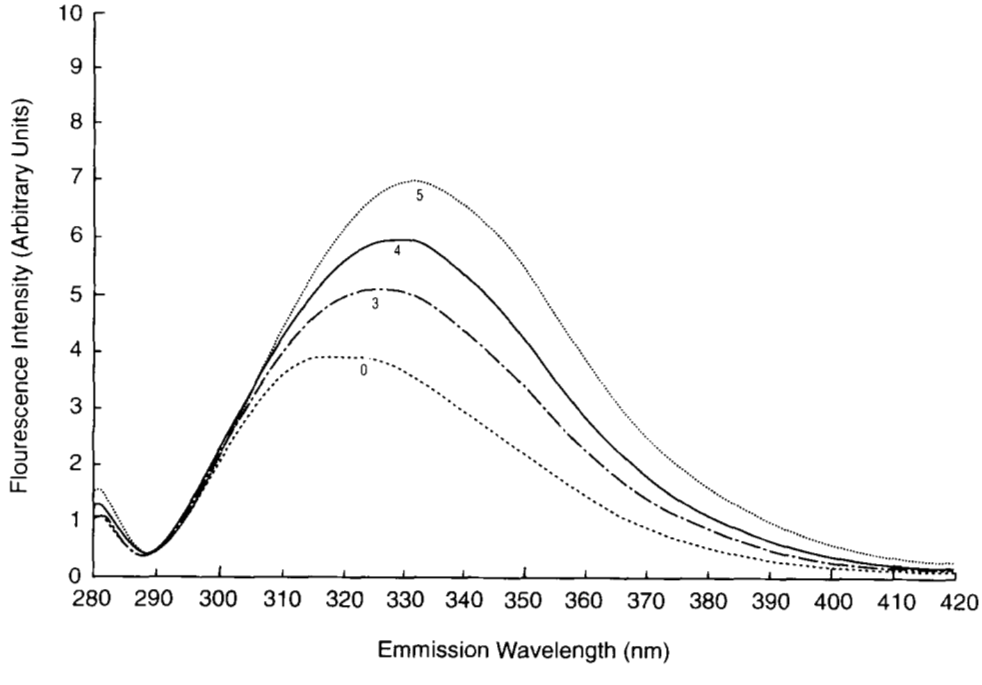

Fig. 6. Fluorescence spectra of native SCF and methionine oxidized species: Native SCF (0); three methionine-oxidized SCF species (3); four methionine oxidized SCF species (4); and five methionine oxidized SCF species (5).

the oxidation of Met^{36}, and lost following the oxidation of Met^{48}, indicating that the Trp^{44} environment is significantly less rigid in this species than in the original sample. However, the environments of the other aromatic amino acids remain unchanged, indicating that this is a local, rather than global, change in the protein fold. The fluorescence spectra also reveal that the Trp^{44} becomes more solvent exposed as the methionines are oxidized, with the hydrophobicity of the Trp^{44} environment decreasing significantly.

Biological activity

The curve for receptor binding competition between the unlabeled SCF sample and I^{125}-labeled SCF was constructed by plotting the percent I^{125}-SCF bound, (B-NSB)/(Bo-NSB), versus the concentrations of each SCF species (Fig. 7). The receptor binding activities of the two or three methionine-oxidized species are identical to that of the native protein. The receptor binding activities of the four and five methionine-oxidized species, as compared at the point of 40% competition, are decreased by more than 30% and 50%, respectively. In the UT-7 mitogenesis assay, the species with two or three methionines oxidized have dose-response curves identical to those of native SCF (Fig. 8). The species with four and five methionines oxidized have less activity. The dose-response curves of these species shift to higher concentrations. Half-maximal activity (1,500 cpm) required 12 ng/mL of native SCF, 20 ng/mL of the species with four methionines oxidized (40% reduction in activity), and 30 ng/mL of the species with five methionines oxidized (60% reduction in activity). These results indicate that oxidation of Met^{36} and Met^{48} reduces mitogenic activity substantially.

Dissociation rate constant k_{-1}

The procedure used to obtain the dissociation rate constant k_{-1} of the SCF dimer is described in the Materials and methods. The oxidized methionine species of SCF(N10D) were obtained by using the same oxidation method described for the native protein, and were used to perform dimer exchange analyzed by cationic exchange SP-5PW HPLC. The dissociation rate constant for each species is listed in Table 2. The species with four and five methionines oxidized have dissociation rate constants twofold and threefold higher than that of native SCF, respectively. This raises the possibility that the oxidation of Met^{36} and Met^{48} affects the dimerization interface. The four and five oxidized methionine species have a lower affinity between the two subunits, as a result of faster dissociation.

Table 2. *Dissociation rate constant k_{-1} for native SCF and three, four, and five methionine oxidized species*

	k_{-1} Dissociation rate constant
SCF wild type	$1.5 \times 10^{-4}\ s^{-1}$
SCF Met (→0) 159, −1, 27	$1.8 \times 10^{-4}\ s^{-1}$
SCF Met (→0) 159, −1, 27, 36	$2.8 \times 10^{-4}\ s^{-1}$
SCF Met (→0) 159, −1, 27, 36, 48	$4.7 \times 10^{-4}\ s^{-1}$

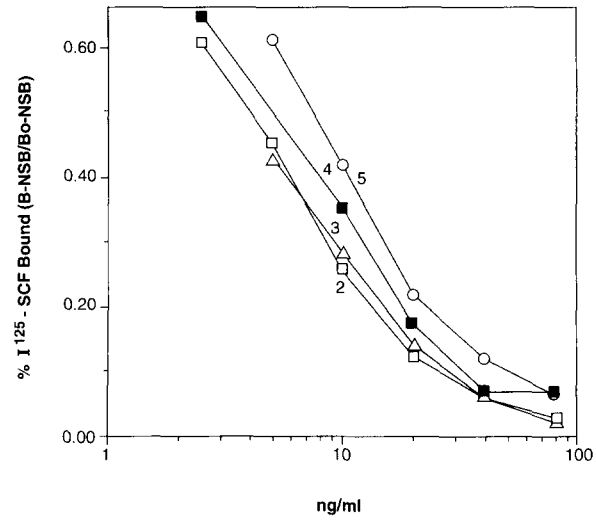

Fig. 7. Comparison of the competition with ^{125}I-SCF for binding to c-kit receptor among the methionine-oxidized SCF species. Dose-response competition curves were plotted as percent I^{125}-SCF bound (B-NSB/B_0-NSB) versus concentrations of each SCF sample. SCF species with two methionines oxidized (□); SCF species with three methionines oxidized (△); SCF species with four methionines oxidized (■); and SCF species with five methionines oxidized (○). B, bound CPM; NSB, nonspecific binding; B_0, bound ^{125}I-SCF at no competition.

Discussion

This study of the in vitro methionine oxidation of SCF suggests that there are three classes of methionine residues in this protein. Met^{-1} and Met^{159}, the first class, are oxidized rapidly and

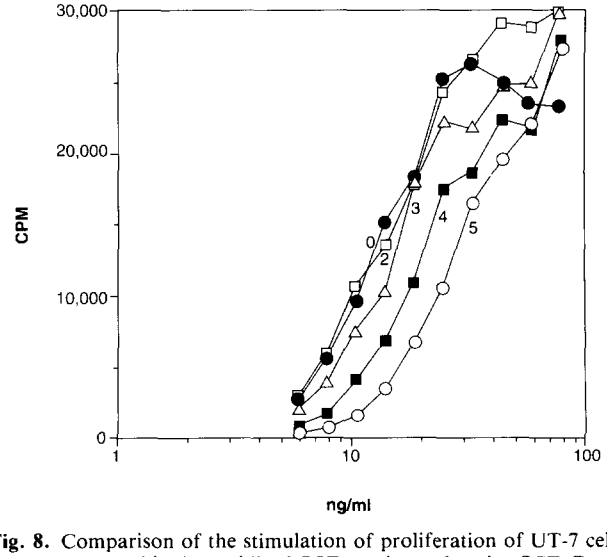

Fig. 8. Comparison of the stimulation of proliferation of UT-7 cells between the methionine-oxidized SCF species and native SCF. Dose-response curves were plotted as CPM versus protein concentration: Native SCF (●); SCF species with two methionines oxidized (□); SCF species with three methionines oxidized (△); SCF species with four methionines oxidized (■); and SCF species with five methionines oxidized (○). CPM, ^{3}H-thymidine incorporation.

appear to be located on the external surface of SCF. Met^{27}, the second class, is oxidized at a slower rate and appears be located in a more rigid, partially solvent-exposed environment. Oxidation of these three methionines does not affect biological activity. This is different than the results obtained with studies of the oxidation of methionine of human growth hormone (hGH) and human chorionic somatomammotropin (hCS) (Teh et al., 1987). For these proteins, the major decrease in the biological mitogenic activity and in the receptor binding activity is clearly associated with the oxidation of one or both of the two most rapidly oxidizing methionine residues, Met^{64} and Met^{179} in hCS, and Met^{125} in hGH. It is suggested that these methionine residues are located on the molecular surface and are a part of the receptor binding sites. In our studies with SCF, the rapidly oxidizing methionyl residues, Met^{-1}, Met^{159}, and Met^{27} are probably located on the surface of the protein, but do not appear to be involved in the receptor binding sites because their oxidation does not affect the biological activity.

The oxidation rates of Met^{36} and Met^{48}, the third class, are 20 and 300 times slower than that of Met^{159}, and the oxidation of these two residues is accompanied by a change in the asymmetry of the environment of Trp^{44} (the only Trp in SCF). The 295-nm Trp peak in the near-UV CD spectra is decreased in the protein with four methionines oxidized, and disappears completely in the protein with five methionines oxidized, indicating that the Trp^{44} environment is increasingly less rigid in these derivatives than in the native SCF. This is accompanied by a red shift in the fluorescence maximum from 320 nm to 330 nm, and also an increase in fluorescence intensity. Thus, the normally hydrophobic environment of Trp^{44} in SCF has become more hydrophilic, more solvent exposed, and less quenched. This result implies that Met^{36} and Met^{48} are buried in a hydrophobic environment.

The oxidation of Met^{36} decreases mitogenic activity by 40%, and oxidation of both Met^{36} and Met^{48} decreases mitogenic activity by 60%. However, if, as we suggest, Met^{36} and Met^{48} are not located on the external surface, they are probably not part of the receptor binding region. Why does their oxidation affect the biological activity? The species with four methionines and five methionines oxidized possess secondary structure identical to that of native SCF, and retain features of the near-UV CD spectra attributable to the other aromatics and the disulfide. These results indicate that the structural change caused by oxidation of Met^{36} and Met^{48} is local rather than global in the protein fold. This is contrary to the results with plasminogen activator inhibitor type 1, where oxidative inactivation resulted from a change in protein secondary structure, as probed by far-UV CD (Strandberg et al., 1991).

Interestingly, the oxidation of both Met^{36} and Met^{48} increases the dissociation rate constant of the SCF dimer by two- to threefold. This suggests that Met^{36} and Met^{48} may be located near the dimerization interface of SCF, or the local perturbation around Trp^{44} caused by the oxidation of Met^{36} and Met^{48} may affect the dimerization interface indirectly. Dimerization of SCF could be driven by hydrophobic interactions. The introduction of an oxygen to methionine increases the polarity of the local environment, and thus may weaken the hydrophobic interaction between the two SCF subunits to some extent. A similar phenomenon has been seen in selective oxidation of methionine β(55)D6 at the $\alpha_1\beta_1$ interface in hemoglobin (Amiconi et al., 1989), as well as in the oxidative dissociation of human α2-macroglobulin tetramers into dysfunctional dimers (Reddy et al., 1994).

When methionine β(55)D6 in human hemoglobin is oxidized to its sulfoxide derivative, the modified protein retains most of the chemical and structural properties of the native protein, but oxygen affinity is decreased drastically. The results reported indicate that the destabilization of the T state of hemoglobin is due to perturbation of the $\alpha_1\beta_1$ subunit interface. In the case of α2-macroglobulin, the antiproteolytic activity was lost due to the oxidation of a subset of methionine and tryptophan residues, causing dissociation of the native homotetramer into conformationally locked dimers.

An X-ray crystallographic structure or NMR structure of SCF is not available. The specificity and selectivity of oxidation of the methionines of SCF provide some structural insight. The rapidly oxidizing methionine residues Met^{-1}, Met^{159}, and Met^{27} appear to be located on the surface of the protein, but not involved in the receptor binding sites. The oxidation of the buried Met^{36} and Met^{48} affects local structure, decreases biological activity, and increases the dimer dissociation rate constant of SCF. We cannot rule out the possibility that local structural change, as reflected by the change in the Trp^{44} environment, results in a change in the surface of the protein at the SCF receptor binding site. We speculate that the biological activities of the four and five methionine-oxidized species could also be lower as a consequence of the higher dimer dissociation rates of these molecules. The dimerization of SCF is possibly required for the dimerization of its receptor c-kit in order to activate signal transduction.

Materials and methods

Materials

Asp-N endoproteinase and trypsin were purchased from Boehringer Mannheim. 4-hydroxy-α-cyanocinnamic acid and hydrogen peroxide were obtained from Aldrich Chemical. Acetonitrile and HPLC grade water were purchased from Burdick & Jackson. All protein sequencing reagents were obtained from Applied Biosystems Inc. Trifluoroacetic acid and other chemicals were purchased from J.T. Baker.

E. coli expression and preparation of recombinant human SCF

The construction of the expression vector for human SCF, the fermentation conditions for high-level expression in *E. coli*, and the purification methods used were reported previously (Martin et al., 1990; Langley et al., 1992). The expressed gene contained an initial methionine codon followed by the codons for SCF 1–165. The purified SCF retained an unprocessed Met at the N-terminus (position −1). The SCF(N10D) analogue was prepared by procedures including site-directed mutagenesis of the parent gene with standard PCR techniques, essentially as described (Erlich et al., 1989; Langley et al., 1992).

Formation and isolation of methionine oxidized SCF species

The SCF preparations in 20 mM sodium acetate, 0.1 M NaCl buffer pH 5.0, were mixed with an equal volume of 0.5%, 1%, or 6% hydrogen peroxide and the mixtures were incubated at

various temperatures and for various durations. The oxidized SCF preparations were then immediately applied to a TSK DEAE-5PW HPLC column (Tosohaas) equilibrated with 20 mM Tris-HCl, pH 7.0, connected to a Hewlett Packard 1050 liquid chromatograph. Hydrogen peroxide eluted in the void volume and SCF eluted during a 10-min gradient from 0 to 0.2 M NaCl in the column buffer at a flow rate of 1 mL/min.

RP-HPLC of SCF

SCF or methionine-oxidized SCF species (50 to 100 μg) were loaded onto a reversed-phase C4 column (Vydac 4.6 × 250 mm) on a Hewlett Packard 1090 HPLC system. The protein was eluted using a gradient of 0.1% trifluoroacetic acid (buffer A) to 90% acetonitrile in 0.1% trifluoroacetic acid (buffer B). The gradient was as follows: an initial equilibration condition of 70% A, 30% B, followed by 5 min of isocratic elution, and then a 10-min linear gradient to 50% B; a 40-min gradient to 70% B; a 5-min gradient to 90% B; and finally, a 5-min isocratic elution in 90% B. The flow rate was 0.7 mL/min.

Asp-N endoproteinase peptide mapping of SCF preparations

Each SCF sample (100 μg) was digested overnight at 37 °C with Asp-N endoproteinase in 20 mM Tris-HCl, pH 7.0, using an enzyme to substrate ratio of 1:75. The digests were added to 10 μL of 0.2 M DTT for 30-min incubation and then subjected to injection onto a reversed-phase C4 column (Vydac 4.6 × 250 mm). The chromatography was performed using a Hewlett Packard 1090 HPLC system equipped with a Chemstation and diode array detector, with a gradient of 0.1% trifluoroacetic acid (buffer A) to 90% acetonitrile in 0.1% trifluoroacetic acid (buffer B). The chromatography was performed with a flow rate of 0.7 mL/min as follows: initial equilibration at 97% A and 3% B; a 5-min gradient to 10% B; a 23-min gradient to 18% B; a 12-min gradient to 28% B; a 24-min gradient to 40% B; a 6-min gradient to 90% B; and a final 10-min isocratic elution at 90% B. The detection wavelength was set at 215 nm.

Trypsin digestion of peptides

The peptide D-Y-M^{27}-I-T-L-K-Y-V-P-G-M^{36}, containing two methionines, was isolated from the Asp-N endoproteinase digests by RP-HPLC, dried in a vacuum centrifuge, reconstituted in 20 mM Tris-HCl, pH 8.0, and then subjected to trypsin digestion for 2 h at 37 °C. The digest was subsequently injected onto an RP-HPLC C4 column and the elution was carried out using the same gradient as described in the Asp-N endoproteinase peptide map.

Sequence analysis and mass spectrometry

Amino acid sequence analysis of intact protein or peptides was performed on an automated ABI protein sequencer (models 477A or 470A) equipped with a model 120A on-line PTH-amino acid analyzer and a model 900A data collection system. Sizes of the purified peptides were determined using matrix-assisted laser desorption mass spectrometry on a Kratos MALDI III instrument. 4-Hydroxy-α-cyanocinnamic acid (5 mg/mL, 0.1% TFA:acetonitrile:ethanol, 65:25:10 (v/v/v)) was used as a matrix. The mass scale was calibrated using peptides of known purity and molecular masses.

Determination of dissociation rate constant k_{-1}

The kinetics of dissociation of the SCF dimer (DD ⇌ D + D) and the equation $k_{-1} = 2Vo/[DD]$ were derived as described in a previous paper (Lu et al., 1995). DD stands for wild-type SCF homodimer, D for wild-type SCF monomer, NN for SCF(N10D) variant homodimer, N for SCF(N10D) monomer, and DN for the heterodimer composed of SCF(N10D) and SCF wild type. The dimer dissociation rate constant k_{-1} was obtained by measuring the initial velocity (Vo) of formation of the heterodimer [DN] using varied initial concentrations of homodimer under conditions where equal amounts of homodimers (DD + NN) were mixed. Equal volumes of the SCF wild type and the SCF (N10D) variant, both at 0.5 mg/mL in 20 mM sodium acetate, pH 4.8, were mixed and then incubated at 25 °C for 5 min, 7.5 min, and 10 min. Fifty microliters of the preparation was then immediately injected onto a cationic exchange SP-5PW HPLC column (Tosohaas) connected to a Hewlett Packard 1050 liquid chromatograph. The elution included a 5-min initial equilibration in 20 mM sodium acetate, pH 4.8 (buffer A) followed by a 30-min gradient to 24% buffer B using a flow rate of 1 mL/min. Buffer B was 0.5 M sodium sulfate in buffer A. Both the buffer and column were held in an ice bath. The initial velocity was determined by the plot of the formation of the heterodimer versus the incubation time within the first-order kinetics. The dissociation rate constant k_{-1} was derived from the equation $k_{-1} = 2Vo/[DD]$. The species of SCF wild type and SCF (N10D) variant with three, four, and five methionines oxidized were analyzed using the same method to obtain a dissociation rate constant for each species.

CD spectroscopy

CD spectra were obtained using a Jasco J-710 spectropolarimeter controlled by a DOS-based system, using cuvettes with a pathlength of 0.02 cm for the far-UV CD region (190–250 nm) and 1.0 cm for the near-UV region (340–240 nm). The spectra were recorded at ambient temperature. The protein concentrations of native SCF and the SCF species with five methionines oxidized were 0.3 mg/mL and the concentrations of other SCF species were 1.5 mg/mL. All the SCF samples were prepared in 20 mM sodium acetate, pH 4.8. Molar ellipticity was calculated assuming a mean residue weight of 112, and protein concentrations were based on the determined A_{280}, assuming an extinction coefficient of 0.62 from an 0.1% protein solution (Arakawa et al., 1991).

Fluorescence spectroscopy

The fluorescence spectra were determined at ambient temperature on an SLM 500C spectrofluorimeter, using a cuvette with a pathlength of 0.5 cm and protein concentrations of 0.22 mg/mL. The solutions were excited at 280 nm, and fluorescence spectra from 280 to 420 nm were recorded, using a slit width of 5 nm.

Biological assay

The biological activity of SCF was determined using an in vitro cell mitogenesis bioassay involving [^{3}H]-thymidine incorpora-

tion by the human megakaryoblastic leukemia cell line UT-7 as described previously (Smith & Zsebo, 1992).

Receptor binding assay

The receptor binding assay was performed as described (Smith & Zsebo, 1992), using membranes prepared from human erythroleukemic cell line OCIM-1, which expresses high levels of c-kit, the SCF receptor.

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