Oxidation of Recombinant Human Interleukin-2 by Potassium Peroxodisulfate

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Purpose. The oxidation of recombinant human interleukin-2 (rhIL-2) by potassium peroxodisulfate (KPS) with or without *N,N,N′,N′*-tetramethylethylenediamine (TEMED), which are used for the preparation of dextran-based hydrogels, was investigated.

Methods. The oxidation of (derivatives of) methionine, tryptophan, histidine and tyrosine, as well as rhIL-2 was investigated. Both the oxidation kinetics (RP-HPLC) and the nature of the oxidation products (mass spectrometry) were studied as a function of the KPS and TEMED concentration, and the presence of a competitive antioxidant, methionine.

Results. Under conditions relevant for the preparation of rhIL-2 loaded hydrogels, only methionine and tryptophan derivatives were susceptible to oxidation by KPS. The oxidation of these compounds was inhibited once TEMED was present, suggesting that the peroxodisulfate anion, rather than the radicals formed in the presence of TEMED, is the oxidative species. KPS only induced oxidation of the four methionines present in rhIL-2, whereas the tryptophan residue remained unaffected. The radicals, formed after KPS decomposition by TEMED, induced some dimerization of rhIL-2. The oxidation of rhIL-2 could be substantially reduced by the addition of methionine, or by pre-incubation of KPS with TEMED.

Conclusions. Only the methionine residues in rhIL-2 are oxidized by KPS. The extent of oxidation can be minimized by a proper selection of the reaction conditions.

KEY WORDS: oxidation; interleukin-2; methionine; peroxodisulfate, mass spectrometry.

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ABBREVIATIONS: ACN, acetonitrile; APS, ammonium peroxodisulfate; PB, 100 mM phosphate buffer, pH 7.2; ESI-MS, electrospray ionization mass spectrometry; His, Nα-acetyl-L-histidine; KPS, potassium peroxodisulfate; NATA, N-acetyl-L-tryptophanamide; rhIL-2, recombinant human interleukin-2; RP-HPLC, reversed-phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEMED, N,N,N′,N′-tetramethylenediamine; TFA, trifluoroacetic acid; Z-Met, Benzyloxocarbonyl-methionine.

INTRODUCTION

Recombinant human interleukin-2 (rhIL-2) is a protein currently under investigation for immunotherapeutic treatments of cancer (1–3). Because of its short half-life *in vivo* (4), suitable controlled release systems are highly required. Crosslinked hydrophilic polymers (hydrogels) form an attractive category of polymeric delivery systems for the controlled release of pharmaceutically active proteins (5). In our department both macroscopic and microscopic dextran-based hydrogels have been extensively studied as protein-releasing systems (6–9). To achieve a sustained release of a protein that is controlled by the degradation of the hydrogel matrix, the initial hydrogel mesh size has to be smaller than the hydrodynamic radius of the protein (8,9). Such gels can be obtained by initiating the polymerization reaction of a protein-containing solution of the methacrylated dextrans by the addition of potassium peroxodisulfate (KPS) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED). The KPS/TEMED initiator system, however, may oxidize proteins, which could result in structural changes and loss of biological activity (10). Among all amino acid residues, those containing a sulfur atom (methionine and cysteine) or an unsaturated ring (histidine, tryptophan and tyrosine) are most susceptible to oxidation (10,11).

In a previous study, the polymerization kinetics of methacrylated dextran was reported with the aim to get a high conversion of methacrylate groups under mild conditions with minimal amounts of KPS and TEMED (12). In the present study, the oxidation of rhIL-2 by KPS and the effect of TEMED hereon were investigated. To gain insight into which oxidation sensitive amino acids (methionine, tryptophan, histidine and tyrosine residues present in rhIL-2) are indeed oxidized by the KPS/TEMED system, the oxidation kinetics of the free amino acids or derivatives thereof were studied by RP-HPLC. Moreover, the oxidation kinetics of a methionine-containing penta-peptide (Ac-Thr-Phe-Met-Ser-Glu-NH_2), corresponding with the surface-exposed sequence containing Met^{104} that is likely to be the most vulnerable oxidation site in rhIL-2 (13), was investigated as well. The nature of the oxidation products was determined by mass spectrometry. Finally, the prevention of the oxidation of rhIL-2 was examined by using L-methionine as a competitive anti-oxidant.

MATERIALS AND METHODS

Materials

Recombinant human interleukin-2 (rhIL-2; Proleukin) was kindly provided by Chiron B.V. (Amsterdam, The Netherlands). This protein differs from native IL-2 in that the protein is not glycosylated, has no N-terminal alanine, and has a serine at position 125 substituted for cysteine.

Benzyloxocarbonyl-methionine (Z-Met) was obtained from Bachem (Bubendorf, Switzerland). Nα-acetyl-L-histidine monohydrate (99+%, His) was purchased from Aldrich (Zwijndrecht, The Netherlands). N-acetyl-L-tryptophanamide (NATA) was obtained from Acros Organics (Geel, Belgium). L-tyrosine (Tyr) was obtained from Baker Chemicals B.V. (Deventer, The Netherlands). Trypsin sequencing grade was purchased from Roche Diagnostic

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(Mannheim, Germany). L-methionine (methionine) was obtained from Merck (Darmstadt, Germany). All other chemicals were obtained from various sources and employed as received.

Solid Phase Synthesis of Penta-Peptide

The penta-peptide, Ac-Thr-Phe-Met-Ser-Glu-NH_2, was synthesized with an Applied Biosystems 433A Peptide Synthesizer, applying the FastMoc protocol on 0.25 mmol scale (14,15). The peptide syntheses were carried out on Argogel-rink-NH-Fmoc (1 g, capacity 0.1 mmol/g) and the product was analyzed with HPLC (purity >95%).

Oxidation of Model Compounds

To investigate the identity of the oxidation products, if any, of Z-Met, NATA, His, Tyr and the penta-peptide, these compounds were dissolved in 100 mM ammonium acetate pH 7.2 (final concentration of 0.5 mM) and incubated for 20 h at 20°C with ammonium peroxodisulfate (APS; 3.7 mM) or APS (3.7 mM) and TEMED (26 mM). APS and ammonium acetate were used instead of KPS and phosphate buffer (PB), respectively, because sodium, potassium, and phosphate ions interfere with electrospray ionization mass spectrometry (ESI-MS). Subsequently, the reaction mixture was analyzed by positive ion ESI-MS. A VG Platform II single quadrupole mass spectrometer was used and aliquots of 10 μL of the sample solution were injected into a mobile phase of acetonitrile (ACN)/water/formic acid (50:50:0.1, v/v/v) and introduced into the electrospray source at a flow rate of 30 μL/min. Spectra were recorded over the m/z 100-800 range using a cone voltage of 40 V and a capillary voltage of 3.25 kV.

Oxidation kinetics of Z-Met, the penta-peptide, and NATA (final concentration 0.5 mM) were studied, except when specified otherwise, at 20°C and in 100 mM PB, pH 7.2. Stock solutions of KPS and TEMED were freshly prepared in the same buffer. Oxidation of the model compounds by KPS was performed by adding KPS stock solution (final concentration varying from 0.25–7 mM) to a solution of the model compound. To study the oxidation of the amino acids/penta-peptide by KPS in the presence of TEMED, first TEMED (final concentration varying from 2 to 56 mM) and subsequently KPS was added (fixed final concentration of 1 mM) to the model compound solution. The effect of the temperature (8, 20, and 37°C) on the oxidation kinetics of Z-Met by KPS was investigated in PB, at a fixed concentration of Z-Met (0.5 mM) and KPS (1 mM).

After mixing, samples were drawn at regular time intervals and analyzed by RP-HPLC. An LC Module I plus system (Waters) with an analytical column (Lichrospher100 RP-18, 5 μm, 125 mm × 4 mm i.d. including an RP-18 guard column (4 × 4 mm; Merck)) was used. Aliquots of 10 or 20 μL were injected and the flow rate was 1.0 mL/min. UV detection at 210 nm was applied and the column temperature was 30°C. For Z-Met and the penta-peptide, a linear gradient was run from 100% A (water/ACN, 95:5, w/w, containing 0.1% trifluoroacetic acid TFA (v/v)) to 100% B (water/ACN, 5:95, w/w, containing 0.1% TFA (v/v)) in 20 min. For NATA, the mobile phase consisted of ACN/water (14:86, w/w) containing 0.1% (v/v) TFA and methanol/water (5:95, w/w) containing 0.1% (v/v) TFA, respectively.

Oxidation of rhIL-2

Oxidation of rhIL-2 (final concentration 17.8 μM) by KPS (0.93–7.4 mM) was carried out at 20°C and in PB containing 0.1% sodium dodecyl sulfate (SDS). The effect of TEMED on the oxidation of rhIL-2 (17.8 μM) was studied at a fixed KPS concentration (3.7 mM) with varying TEMED concentrations (12, 24, and 48 mM). TEMED followed by KPS were added to an rhIL-2 solution. Alternatively, TEMED and KPS (3.7 and 24 mM, respectively) were mixed and incubated for 60 min, and subsequently added to the protein solution. Also, the influence of Met (final concentration 7.4 or 29.6 mM) on the oxidation of rhIL-2 (17.8 μM) by KPS (3.7 mM) and TEMED (48 mM) was studied.

After mixing, samples were drawn at regular time intervals and analyzed by RP-HPLC (16). rhIL-2 samples were analyzed by RP-HPLC. A LC Module I system (Waters) with an analytical column (Jupiter, 5 μm C4 300 A, 150 × 4.6 mm including a SecurityGuard guard cartridge system with Widepore C4 (4 × 3 mm)) was used. A linear gradient was run from 40% A (water/acetonitrile 95:5; w/w; 100 mM sodium perchlorate ($NaClO_4$); 10 mM perchloric acid ($HClO_4$)) and 60% B (water/acetonitrile 5:95; w/w; 100 mM $NaClO_4$; 10 mM $HClO_4$) to 100% B in 10 min. The flow rate was 1.0 mL/min and the column oven was set at 30°C. UV detection at a wavelength of 205 nm was applied.

For ESI-MS, ACN/water containing 0.1% (v/v) TFA was used as eluent instead of ACN/water with 100 mM $NaClO_4$ and 10 mM $HClO_4$, since sodium ions interfere with MS analysis. A linear gradient was run from 50% A (water/ACN 95:5 (w/w) containing 0.1% TFA) and 50% B (water/ACN 5:95 (w/w) containing 0.1% TFA) to 80% B in 20 min. Peaks corresponding to rhIL-2 and its oxidized products were collected and lyophilized. The obtained products were dissolved to a concentration of 0.3–10 μM in ACN/water (50:50 (v/v)) containing 0.1% formic acid and subsequently characterized by positive ion nano ESI-MS on a Micromass quadrupole time of flight (Q-ToF) mass spectrometer equipped with a Z-spray ion source. The gold coated capillaries were loaded with 2 μL sample solution and a potential of 1600 V was applied to it. Mass spectra were recorded over the m/z 500–3000 range using a cone voltage of 50 V.

Digestion of rhIL-2 by Trypsin

Untreated rhIL-2, KPS treated rhIL-2 and KPS/TEMED treated rhIL-2 (concentration of rhIL-2, KPS and TEMED 17.8 μM, 1.85 μM, and 24 mM, respectively, 1 h incubation) were fractionated by RP-HPLC and lyophilized. The lyophilized rhIL-2 fractions were digested with trypsin in 100 mM ammonium hydrogencarbonate, pH 8 (molar ratio trypsin:rhIL-2 1:50) for 20 h at ambient temperature. The reaction mixtures were frozen (–20°C) and analyzed by nano ESI-MS. Percentages of oxidized species were estimated from the relative peak areas.

Gel Electrophoresis

SDS-PAGE was performed under reducing conditions with a 12.5% separating gel containing 0.4% SDS. Samples were heated for 5 min. at 100°C in the presence of 1% (v/v) mercaptoethanol. The applied amount of protein was 10 μg/slot. As control, rhIL-2 containing 6% of dimer was used

(kindly provided by Chiron B.V., Amsterdam, The Netherlands).

RESULTS

Oxidation Kinetics of Z-Met and Penta-Peptide

First, the oxidation by KPS was studied of Z-Met and a methionine-containing penta-peptide (Ac-Thr-Phe-Met-Ser-Glu-NH_2), which corresponds with the surface-exposed sequence containing Met^{104} that is likely to be most susceptible to oxidation (13). After addition of KPS to Z-Met, the RP-HPLC chromatograms showed two peaks, corresponding to the oxidized product Z-Met sulfoxide and Z-Met, respectively, as confirmed by ESI-MS. Even after prolonged incubation periods and at relatively high KPS concentration, Z-Met sulfoxide was the only oxidation product. Similarly, the penta-peptide was only converted to the mono-oxidized species (Ac-Thr-Phe-MetOx-Ser-Glu-NH_2).

When plotting the natural logarithm of the residual Z-Met or penta-peptide fraction vs. time, a linear correlation was obtained, indicating the kinetics of the oxidation reaction to be pseudo first order. From the slopes, the k_{obs}-values were calculated and plotted vs. the KPS concentration. As shown in Fig.1, k_{obs} is linearly proportional to the KPS concentration, which demonstrates that oxidation of Z-Met/penta-peptide is first order in KPS. As Z-Met and the penta-peptide are oxidized almost at the same rate (Fig. 1), the neighboring amino acids in the penta-peptide have a negligible effect on the accessibility of the methionine residue to KPS.

We also studied the temperature dependency of the oxidation of Z-Met by KPS. The activation energy as derived from an Arrhenius plot was calculated to be 44 ± 3 kJ/mole. This is in the order of magnitude of methionine oxidation in relaxin by hydrogen peroxide (17).

Figure 2 shows the effect of TEMED on the oxidation rate of Z-Met at a fixed KPS concentration. Comparable results were obtained for the oxidation of the penta-peptide. This figure illustrates that the initial oxidation rate of Z-Met is not affected by TEMED. After a certain time, which decreases with increasing TEMED concentration, the oxidation levels off and finally stops. At the highest TEMED concentration investigated, the oxidation of Z-Met is only marginal.

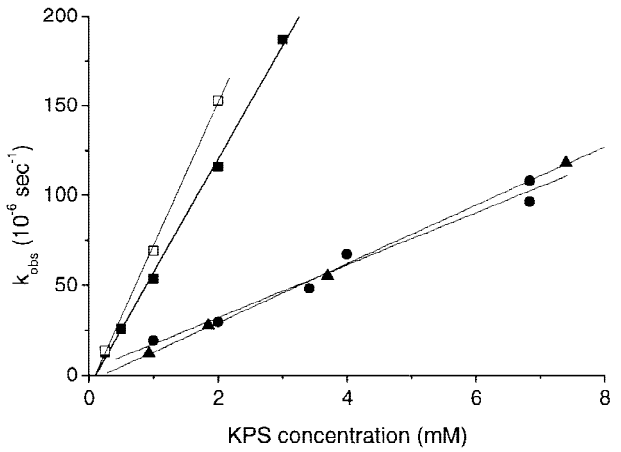

Fig. 1. k_{obs}-values vs. potassium peroxodisulfate (KPS) concentration for the oxidation of Z-Met (■), penta-peptide (□), NATA (●), rhIL-2 (▲) with KPS at pH 7.2 and 20°C. The intra- (n = 3) and interday (n = 5) variation in k_{obs} was 2.4 and 6.4%, respectively.

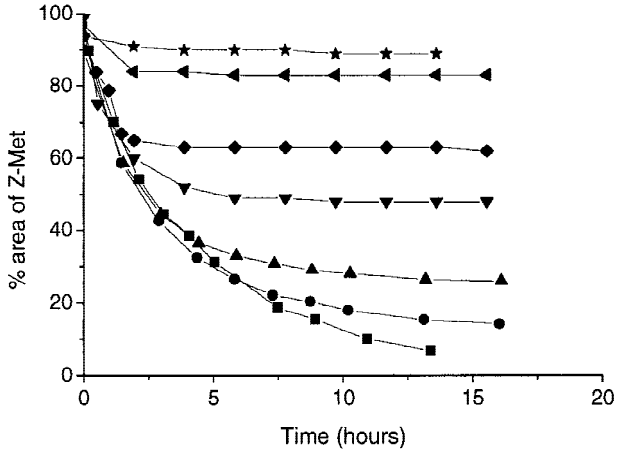

Fig. 2. Effect of TEMED on the oxidation of Z-Met (0.5 mM) by KPS (1 mM) at a varying TEMED concentration (0 mM (■); 2 mM (●); 3.5 mM (▲); 7 mM (▼); 14 mM (◆); 28 mM (◀) and 56 mM (★)) at pH 7.2 and 20°C.

Oxidation of NATA, His, and Tyr

Similar to the oxidation of Z-Met/penta-peptide, the oxidation of NATA at different KPS concentrations followed first order kinetics. Figure 1 shows that k_{obs} is linearly proportional to the KPS concentration, which demonstrates that oxidation of NATA is first order in KPS. The second order reaction rate constant of NATA ((14.5 ± 1.0) × 10^{-6} sec^{-1} mM^{-1}) is approximately a factor 4 lower than that of Z-Met by KPS ((63.0 ± 2.0) × 10^{-6} sec^{-1} mM^{-1}), confirming that tryptophan is less susceptible to oxidation than methionine (18).

As observed for Z-Met and the penta-peptide, the oxidation of NATA by KPS is prevented by adding a high concentration of TEMED. This indicates that the peroxodisulfate anion acts as oxidizing species for NATA, without contribution of the radicals formed after the decomposition of the anion by TEMED (Fig. 3). Mass spectrometry showed that as oxidation products were formed the mono-oxidized (N-acetyl oxindolylalanine amide; m/z = 262) and some traces of the dioxidized product (N-acetyl dioxindolylalanine amide; m/z

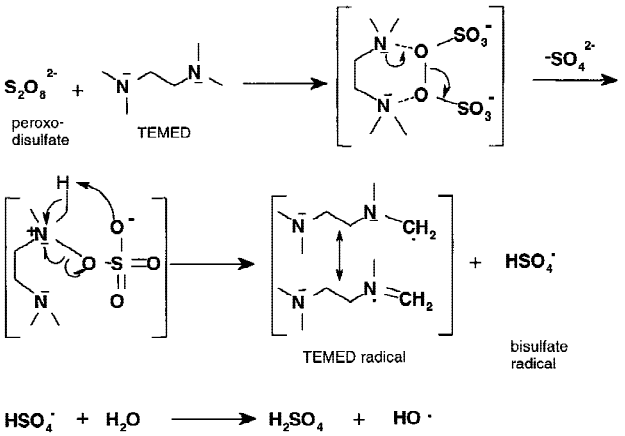

Fig. 3. Formation of radicals from peroxodisulfate and TEMED (28).

= 278). Oxindolylalanine is also an oxidation product of tryptophan treated with hydrogen peroxide (19).

In contrast to the methionine and tryptophan residues, His and Tyr were oxidized by neither the peroxodisulfate anion in the concentration range where substantial oxidation of methionine and tryptophan occurs, nor by the radicals generated by TEMED.

Oxidation of rhIL-2

Figures 4A and 4B show representative RP-HPLC chromatograms of untreated rhIL-2 and rhIL-2 exposed to KPS, respectively. For KPS-treated rhIL-2, two peaks were obtained with retention times of 11.5 (peak *1*) and 12 min (peak *2*), whereby peak *1* increased in time with a concomitant decrease of peak *2*. Furthermore, a small peak with a retention time of 13.5 min (peak *3*, Fig. 4B) was detected which did not change in time. The total area under these peaks corresponded to the initial area, indicating a quantitative recovery.

Nano ESI-MS showed that untreated rhIL-2 (Fig. 5A) contained, besides native rhIL-2 (M = 15326), also some oxidized species, especially mono-oxidized rhIL-2 (M = 15342). Peak *2* of KPS-treated rhIL-2 (see Fig. 4B) consisted of native rhIL-2, mono-, di- (M = 15358) and minor tri- (M = 15374) and tetra-oxidized (M = 15390) species (Fig. 5B). This is in contrast to peak *1* of KPS-treated rhIL-2, which only contained mono-, di-, tri- and tetra-oxidized rhIL-2 products (Fig. 5C).

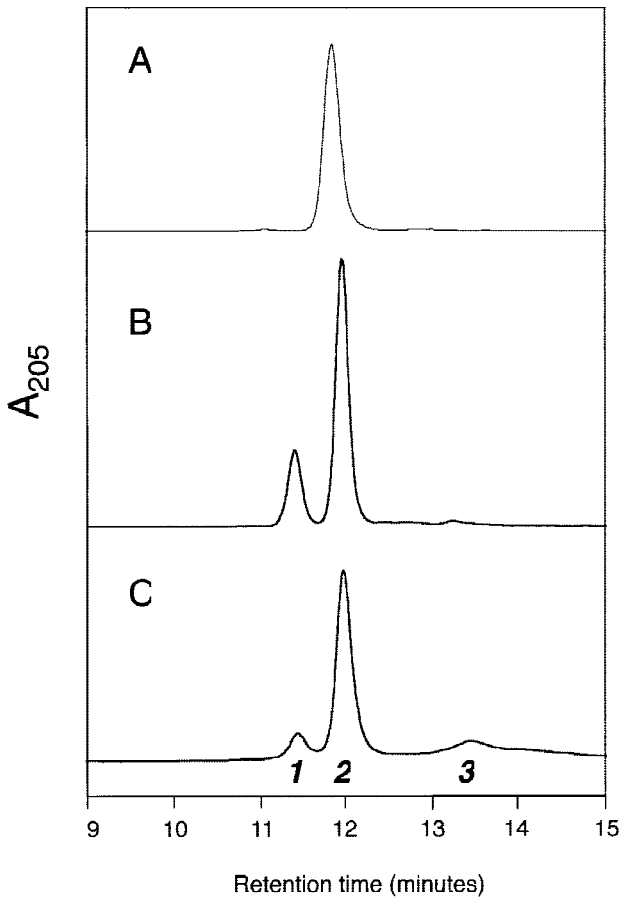

Fig. 4. RP-HPLC chromatograms of untreated rhIL-2 (A), of rhIL-2 (17.8 μM) with KPS (B) and KPS with TEMED (C) after 1 h incubation at ambient temperature. Concentrations of KPS and TEMED are 3.7 and 24 mM, respectively.

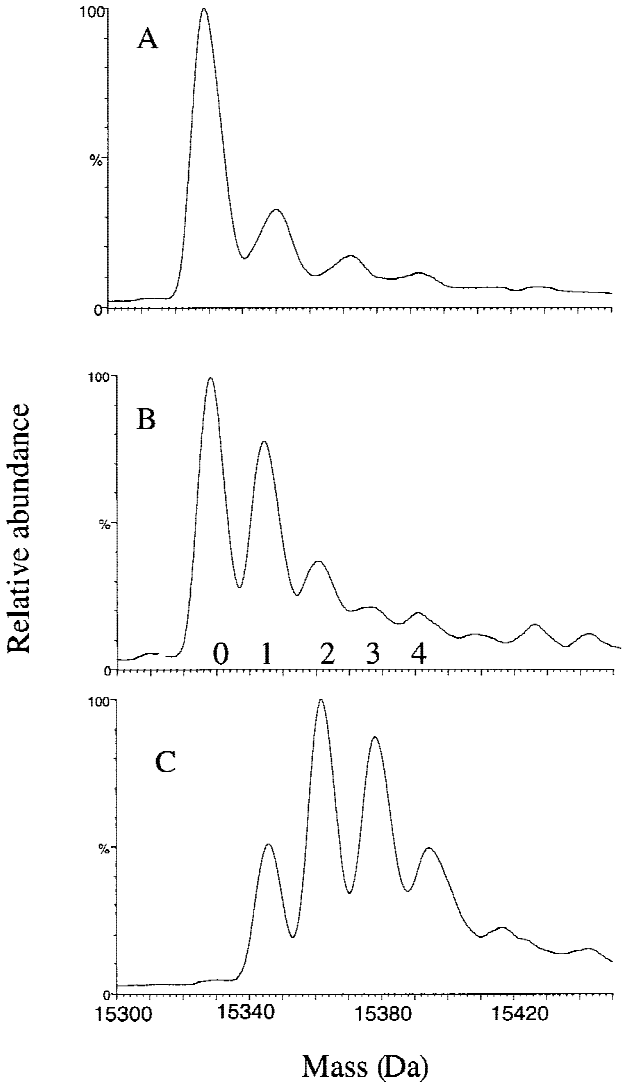

Fig. 5. Nano ES-MS of the RP-HPLC peak *2* (see Fig. 4) of untreated rhIL-2 (A) and the RP-HPLC peak *2* (B) and peak *1* (C) of rhIL-2 (17.8 μM) incubated with KPS (1.85 mM) for 1 h. The numbers 0-4 correspond to a mass of 15326, 15342, 15358, 15374 and 15390, respectively.

Peak *1* and *2* of KPS-treated rhIL-2 and, as control, untreated rhIL-2 were digested with trypsin. Table I summarizes the results. Nano ESI-MS analysis of the fragments of the rhIL-2 standard showed that methionine residues in this protein were already oxidized for 10–15% (Table I). This is consistent with the results shown in Fig. 5A and can probably be attributed to oxidation of the protein during formulation and/or storage. As shown in Table I, peak *1* of KPS-treated rhIL-2 contained rhIL-2 in which all methionine residues at position 104 (Met^{104}) were oxidized, whereas the methionine residues at the other positions were oxidized for 57–66%. Obviously, once the surface-exposed Met^{104} is oxidized, the protein becomes more hydrophilic, which in turn explains the shorter retention time of Met^{104}-oxidized rhIL-2.

Peak *2* (12 min.) contained rhIL-2 in which ca. 60%, 36%, 38%, and 29% of Met^{23}, Met^{39}, Met^{46}, and Met^{104},

Table I. Tryptic Fragments of Untreated rhIL-2, KPS-Treated rhIL-2 and KPS/TEMED-Treated rhIL-2

			Untreated		KPS-treated				KPS/TEMED-treated	
			peak *2*		peak *1*		peak *2*		peak *2*[a]	
Fragment[b]	Oxidative sensitive residue	M^c_{ther}	M^d_{det}	% Ox.[e]	M^d_{det}	% Ox.[e]	M^d_{det}	% Ox.[e]	M^d_{det}	% Ox.[e]
2–8	—	707.36	707.3		707.3		707.3		707.3	
10–32	Met^{23}	2724.48	2724.3		2724.2		2724.2		2724.2	
			2740.3	13	2740.2	57	2740.2	60	2740.2	33
39–43	Met^{39}	639.35	639.3		639.3		639.3		639.3	
			655.3	11	655.3	64	655.3	36	655.3	22
44–48	Met^{46}	685.34	685.3		685.3		685.3		685.3	
			701.3	11	701.3	66	701.3	38	701.3	27
50–54	—	561.32	561.3		561.3		561.3		561.3	
55–76+	Met^{104}	5188.50	5188.1				5188.0		5188.1	
98–120[f]			5204.1	14	5204.0	100	5204.0	29	5204.1	27
77–83	—	939.53	939.4		939.4		939.4		939.5	
84–97	—	1582.94	nd		nd		nd		nd	
121–133	Trp^{121}	1496.80	1496.7		1496.7		1496.6		1496.6	

[a] only this peak could be analyzed, since the concentration of the other peaks was too low.
[b] fragments shown with Mw >500 Da; numbers refer to amino acid sequence in rhIL-2.
[c] theoretical mass of the fragment.
[d] detected mass of the fragment.
[e] percentage of oxidized compound based on peak heights.
[f] fragments linked via S-S bridge.
nd: not detected; peak numbers refer to those in Fig. 4.

respectively, were oxidized. This is in contrast with the results of Sasaoki *et al*., who reported that Met^{23} and Met^{39} (in this order) are oxidized slowly after preferential oxidation of Met^{104}, with no detectable oxidation of Met^{46} (13). This can be explained by the slightly higher redox potential of the peroxodisulfate anion (+2.00 V) compared to H_2O_2 (+1.77 V) (20) and possibly by other differences in experimental conditions (13).

ESI-MS of the digest of peak *1* demonstrated that all species present in peak *1* of the HPLC chromatogram of KPS-treated rhIL-2 (Fig. 4B) contained oxidized Met^{104}. Therefore, the conversion of rhIL-2 into Met^{104}-oxidized rhIL-2 can be quantitatively analyzed by plotting the natural logarithm of the area under the curve of peak *2* vs. time. Figure 1 shows that the so obtained apparent k_{obs}-value was linearly proportional to the KPS concentration. The second order rate constant was a factor four smaller than found for the methionine derivatives (Fig. 1). Because SDS present in the buffer is likely to interact with rhIL-2, the protein becomes negatively charged. The negative surface charge might repel the peroxodisulfate anion, resulting in a slower oxidation rate of Met^{104} by KPS.

The oxidation of rhIL-2 by KPS/TEMED was studied at a fixed KPS and varying TEMED concentrations. As shown in Fig. 4C, three peaks were obtained by RP-HPLC with retention times of 11.5, 12 (both observed for KPS treated rhIL-2; Fig. 4B) and 13.5 min, respectively. In contrast to KPS-treated rhIL-2, peak *1* (11.5 min.) reached a plateau value within 30 min, whereas the area of peak *2* (12 min.) decreased concomitantly with an increase of the area of peak *3* (13.5 min.; Fig. 6, closed symbols).

The data were kinetically analyzed by plotting the natural logarithm of the area under the curve of peak *2* vs. time (Fig. 7). In contrast to the KPS-induced oxidation of rhIL-2, the oxidation of this protein by KPS/TEMED did not follow pseudo first order kinetics. The initial oxidation rate was not dependent on the TEMED concentration and faster than the oxidation observed by KPS only. After a certain time (1–4 h, dependent on the TEMED concentration) the oxidation rate decreased. Moreover, by increasing the TEMED concentration the area of peak *1* was hardly affected. Importantly, the residual amount of 'native' rhIL-2 (peak *2*) decreased to a lesser extent with increasing amount of TEMED. Thus, rhIL-2 is less affected at higher TEMED concentrations.

SDS-PAGE analysis showed that covalently bonded dimers of rhIL-2 were formed after incubation of rhIL-2 with KPS and TEMED. Moreover, after 6 h of incubation with KPS and TEMED minor amounts of (covalently bonded) ag-

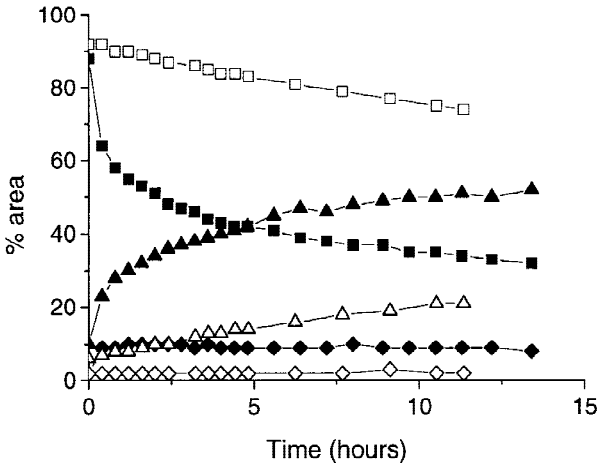

Fig. 6. Percent of area of peak *1* (◆,◇), *2* (■,□) and *3* (▲,△) (Fig. 4C) as a function of time after incubation of rhIL-2 (17.8 μM) with KPS (3.7 mM) and TEMED (24 mM) (closed symbols) or after addition of the pre-incubated mixture of KPS/TEMED to rhIL-2 (open symbols).

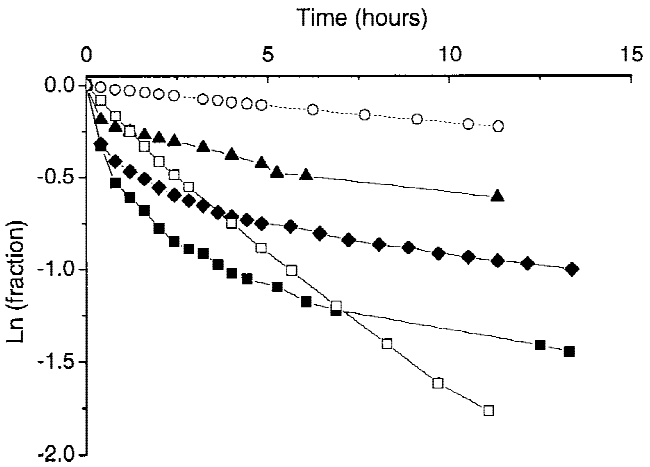

Fig. 7. The effect of TEMED on the oxidation rate of rhIL-2 (17.8 μM; ln (fraction of the area of peak *2*) vs. time) by KPS (3.7 mM) at a varying TEMED concentration (0 mM (□); 12 mM (■); 24 mM (◆) and 48 mM (▲); degradation of rhIL-2 (17.8 μM) after addition of a mixture of KPS (3.7 mM) and TEMED (24 mM) pre-incubated for 60 min. (○).

gregates of rhIL-2 were formed. SDS-PAGE of HPLC fractions of rhIL-2 demonstrated that the dimers were mainly accumulated in peak *3*, which was confirmed by the nano ESI-MS analysis of this fraction (not shown). On the other hand, no significant aggregation was observed for KPS-treated rhIL-2 in the absence of TEMED.

Prevention of Oxidation

Methionine was evaluated as anti-oxidant (21) for the KPS/TEMED-induced oxidation of rhIL-2. It was shown by RP-HPLC that the amount of Met^{104}-oxidized rhIL-2 formed after 1 h incubation (rhIL-2, KPS and TEMED concentrations of 17.8 μM, 3.7 mM and 48 mM, respectively) was significantly reduced in the presence of methionine (8, 5, and 3% oxidized Met^{104} (based on peak *1*) for 0 mM, 7.4 mM and 29.6 mM methionine, respectively). Obviously, a large excess of methionine prevents oxidation of the methionine residues in rhIL-2 against oxidation by KPS. Methionine also protects rhIL-2 against radical-mediated degradation. This is surprising, since methionine is not reactive with the radicals formed by the TEMED-catalyzed decomposition of the peroxodisulfate anion (Fig. 2). A likely explanation is that due to the reaction of methionine with KPS, the concentration of the anion decreases. This in turn will result in a decreasing amount of radicals formed, yielding a decreased radical-mediated alteration of rhIL-2.

DISCUSSION

This study shows that under conditions relevant for the preparation of rhIL-2 loaded hydrogels, only the four methionine residues present in rhIL-2 are susceptible to oxidation by KPS. Interestingly, the oxidation is inhibited once TEMED is present. De Feng *et al.* suggested that TEMED accelerates the homolytic scission of the peroxodisulfate anion resulting in the bisulfate free radical, $HSO_4^{\cdot}$ (22). Moreover, the TEMED free radical and the hydroxyl free radical $HO^{\cdot}$ are generated (Fig. 3). It cannot be excluded that besides the radicals shown in Fig. 3 other radicals are formed. For instance, a TEMED radical may reduce peroxodisulfate and induce peroxodisulfate decomposition in a chain process. Whatever role TEMED exactly plays, the results of Figure 2 suggest that the peroxodisulfate anion, and not the radicals formed in the presence of TEMED, is the oxidative species. This conclusion was supported by the fact that the addition of KPS to a mixture of Z-Met, KPS and TEMED, after a plateau value of Z-Met was reached, resulted in a further oxidation of Z-Met, at a rate comparable to the initial rate (data not shown).

Importantly, although NATA was sensitive toward oxidation by KPS (Fig. 1), we did not detect any oxidation of Trp^{121} by KPS (Table I). Likely, the tryptophan residue is buried in the protein structure and, consequently, not accessible to the oxidative species. This conclusion is supported by previous fluorescence studies, which showed that Trp^{121} in rhIL-2 has a relatively hydrophobic environment (emission maximum 335 nm) and is hardly accessible to the quencher iodide (a bulky anion, like peroxodisulfate) (23). Since only methionine-containing fragments showed a mass difference of 16 (Table I), and His and Tyr were found to be resistant to oxidation by KPS under the experimental conditions, we conclude that the histidine and tyrosine residues in rhIL-2 were not oxidized by KPS.

On incubation with KPS only mono-oxidized Met residues were formed in Z-Met, the pentapeptide, and rhIL-2. Methionine sulfoxide as single oxidation product was also obtained after incubation of methionine with hydrogen peroxide (17) and peroxynitrite (24). The dioxidized form, methionine sulfone, most likely is only formed under drastic oxidative conditions, e.g., 95% performic acid (25).

Whereas for the model compounds the oxidation fully stopped once the peroxodisulfate anion was converted into radicals, for rhIL-2 a radical-induced conversion still occurs (a plateau was not obtained; Fig. 7, open circles). Once KPS and TEMED were pre-incubated (60 min) before addition to rhIL-2, only a marginal peak *1* was observed (Fig. 6, open symbols), demonstrating that Met^{104} oxidation was prevented. On the other hand, peak *3* increased in time (Fig. 6, open triangles). This suggests that this peak is due to a radical-induced degradation of rhIL-2. Figure 7 also shows that the reaction rate in the second phase of the degradation of rhIL-2 by KPS and varying TEMED concentrations is comparable to the degradation rate of rhIL-2 when KPS and TEMED are pre-incubated. This strengthens our impression that at this stage radical-induced degradation of rhIL-2 occurs.

Taking the data of the amino acid derivatives and rhIL-2 into account, it seems that two degradation mechanisms occur when rhIL-2 is incubated with KPS in the presence of TEMED. In the initial stage, a combination of KPS-induced oxidation and radical-induced aggregation occurs, resulting in a faster overall degradation rate of rhIL-2 by KPS/TEMED than by KPS only (Fig. 7). Once the peroxodisulfate anion is converted into radicals, no further oxidation of methionine residues in rhIL-2 takes place, as evidenced from the results with methionine derivatives (Fig. 2) as well as the constant concentration after a certain of time of Met^{104}-oxidized rhIL-2 (Fig. 6, curve of peak *1*). In the second stage, further degradation of rhIL-2 occurs by radicals formed by the TEMED catalyzed decomposition of the peroxodisulfate anion (Fig. 3). It is difficult to say which amino acid residues are involved in the radical-mediated aggregation, since many

amino acid residues can potentially react with a highly reactive radical such as the hydroxyl radical (26).

The extent of oxidation of rhIL-2 by the KPS/TEMED initiator system can be reduced by decreasing the KPS concentration, by decreasing the polymerization time and by increasing the TEMED concentration. Importantly, oxidation of rhIL-2 can be prevented by an anti-oxidant, such as methionine.

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