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REVERSED-PHASE CHROMATOGRAPHY OF INTERLEUKIN-2 MUTEINS

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SUMMARY

Human recombinant interleukin-2 (IL-2) and related species have been characterized by chemical modifications, tryptic digestion, and cyanogen bromide digestion. The oxidation states of the cysteines and methionines in several IL-2 muteins* have been determined. Reversed-phase high-performance liquid chromatography allowed us to distinguish the modifications in these muteins and to correlate retention behavior with their structure.

INTRODUCTION

Human interleukin-2 (IL-2), a lymphokine of *ca.* 15 000 dalton, is a potent immunomodulator which is currently being evaluated in clinical trials for anti-tumor activity and Acquired Immune Deficiency Syndrome therapy. IL-2 is a hydrophobic protein with limited aqueous solubility, which can be purified by reversed-phase chromatography with aqueous acetonitrile and trifluoroacetic acid as eluents[1]. Previous structural studies on recombinant and native IL-2 have established the location of the single disulfide linkage through the use of peptide mapping[2,3]. They have also revealed N-terminal heterogeneity as a result of incomplete N-terminal post-ribosomal processing in *Escherichia coli*[2]. We have now confirmed these results and determined the oxidation states of the methionines and cysteines in IL-2 species resolvable by reversed-phase high-performance liquid chromatography (RP-HPLC). Previous investigators have utilized this structural information to successfully reduce problems such as N-terminal heterogeneity, incorrect disulfide bridging, and the initial site of methionine oxidation by producing IL-2 muteins[4–8]. These IL-2 muteins have resulted in improving the protein's production, stability, and potential effectiveness as a human therapeutic. We have characterized the structural differences between these IL-2 muteins by correlation to their RP-HPLC retention.

* The term "mutein" describes a genetically engineered protein expressed from a nucleic acid sequence which has been altered using techniques such as site-specific mutagenesis. Such genetic alterations are designed to result in one or more substitutions, additions, or deletions to the amino acid sequence of the parent protein.

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EXPERIMENTAL

Apparatus

Chromatography of tryptic digests was performed with System 1 consisting of a SP 8700 pump (Spectra Physics, San Jose, CA, U.S.A.) a WISP 710B injector (Waters, Milford, MA, U.S.A.) an Altex 155-40 detector (Beckman, Berkeley, CA, U.S.A.), and either a Model 3390 integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.) or a Nelson 6000 data system (Nelson Analytical, Cupertino, CA, U.S.A.). The 250 × 4.6 mm I.D. RP-300 column and 30 × 4.6 mm I.D. RP-300 pre-column (Brownlee, Santa Clara, CA, U.S.A.) were heated to 30°C in a column block heater (Jones Chromatography, Columbus, OH, U.S.A.). The eluents, used at a flow-rate of 1.0 ml/min were: 0.1% trifluoroacetic acid (TFA) in water, 5% acetonitrile–0.1% TFA, and 0.1% TFA in acetonitrile. Two elution gradients were produced by System 1. Gradient 1: 0% acetonitrile at 0 min, 5% acetonitrile at 5 min, 22.1% acetonitrile at 18 min, 44.9% acetonitrile at 36 min, 80.0% acetonitrile at 55 min and 0% acetonitrile at 60 min, followed by a 15-min equilibration. Gradient 2: 5% acetonitrile at 0 min, 18.3% acetonitrile at 5.5 min, 22.1% acetonitrile at 13 min, 44.9% acetonitrile at 31 min, 80% acetonitrile at 50 min and 5% acetonitrile at 55 min, followed by a 15-min equilibration.

IL-2 muteins were chromatographed with Systems 2 and 3. System 2 consisted of an Altex 322 chromatograph with a WISP 710B injector, a Nelson 6000 data system and a 5-μm 250 × 4.6 mm I.D. 214TP column (Vydac, Hesperia, CA, U.S.A.) which was heated to 30°C in the solvent column block heater. The eluents were: solvent A, 10% acetonitrile–0.1% TFA; solvent B, 0.1% TFA in acetonitrile. The elution gradient (Gradient 3) was linear from 35% to 60% acetonitrile over 40 min, followed by a 10-min equilibration. The flow-rate was 2.0 ml/min. System 3 consisted of a SP 8700 pump, a WISP 710B injector, a Spectroflow 773 detector (Kratos, Ramsey, NJ, U.S.A.) a 650-10S fluorometer (ex. 280 nm, em. 350 nm) (Perkin-Elmer, Mountain View, CA, U.S.A.) and a Nelson 6000 data system. The column, identical with the Vydac column listed above for System 2, but with the addition of a 30 × 2 mm I.D. pre-column packed with the same material, was maintained at 30°C in the column block heater. The eluents were also the same as in System 2. The elution gradient (Gradient 4) was 10% acetonitrile at 0 min, 40.6% acetonitrile at 12 min, 70.3% acetonitrile at 45 min, 100% acetonitrile at 50 min, 10% acetonitrile at 55 min, followed by a 7-min equilibration. The flow-rate was 1.0 ml/min. In all three systems the absorbance at 214 nm was used for the detection of proteins.

Protein and peptide sequencing were carried out by automated Edman degradation, employing a 890C spinning-cup sequencer (Beckman Instruments, Irvine, CA, U.S.A.) and the Beckman program No. 121078. Amino acid compositions were determined with a 121 MB analyzer (Beckman) and Beckman reagents were used for both instruments. The proteins were concentrated in a Speedvac apparatus (Savant, Hicksville, NY, U.S.A.).

Materials

The IL-2 muteins were obtained from the Molecular Biology, Fermentation and Development, and Protein Chemistry Departments of Cetus Corporation (Emeryville, CA, U.S.A.)[4–9]. Chloramine T, dithiothreitol (DTT), Tris buffer and iodoac-

etate were obtained from Sigma (St. Louis, MO, U.S.A.); sodium dodecyl sulfate (SDS) from Bio Rad (Richmond, CA, U.S.A.); Sephadex G-25 from Pharmacia (Piscataway, NJ, U.S.A.); urea from Schwartz-Mann (Cambridge, MA, U.S.A.); TPCK-treated trypsin from Boehringer-Mannheim (Indianapolis, IN, U.S.A.); acetonitrile and formic acid from J. T. Baker (Phillipsburg, NJ, U.S.A.); trifluoroacetic acid, which was redistilled, from Aldrich (Milwaukee, WI, U.S.A.); and cyanogen bromide from Pierce (Rockford, IL, U.S.A.).

Methods

IL-2 was isolated and the cystine disulfide was generated by a proprietary oxidation process[8,9]. IL-2 protein Peaks A and B were fractionated on a RP-HPLC column until more than 95% pure, then dialyzed against a 0.1% SDS–0.05 *M* phosphate solution (pH 7.0), and concentrated to 0.5 mg/ml in the Speedvac. Methionine in IL-2 was oxidized by the addition of 1–50 molar equivalents of chloramine T, buffered with 1.5 *M* Tris (pH 8.8). The reaction mixture was incubated for 2 min to 1 h at room temperature. To reduce cystine-containing IL-2, DTT was added to 10 m*M*, the pH was adjusted to 8–9 with Tris buffer, and the mixture was incubated for 30 min at 60°C. When chloramine T was present, the DTT concentration was adjusted to 20 m*M*. A 10:1 (w/w) ratio of iodoacetate to IL-2 was used to carboxymethylate reduced cysteines. The mixture was incubated in Tris buffer (pH 8–9) for 1 h at room temperature.

Because trypsin activity is lost in the presence of high concentrations of SDS and DTT, a 20 × 0.7 cm I.D. Sephadex G-25 column was used to remove both compounds from 0.25 mg of IL-2 protein. A 2 *M* urea–50 m*M* Tris buffer (pH 8.1) was used to maintain protein solubility in the column, and the protein was monitored at 280 nm. The protein-containing fractions were pooled and assayed for SDS by the method of Sokoloff[10]. If the SDS concentration was more than 20 ppm, the protein was rechromatographed under the same conditions. A trypsin solution (50 μg) in the above buffer was added to the IL-2 at three 45-min intervals and incubated at 37°C for a total of 4 h. Approximately 50 μg of the resulting peptides were injected into the RP-300 column and eluted with Gradient 1 or 2.

RESULTS AND DISCUSSION

RP-HPLC gave evidence of minor impurities in some IL-2 preparations. These impurities have been formed under non-reducing conditions as well as after extended liquid storage[9]. One minor component (Peak A) retains biological activity and has an N-terminal amino acid sequence and composition identical with that of the primary component (Peak B in Fig. 1). It has been found that oxidation of the cystine-containing material in Peak B with chloramine T will productin Peak A[9]. Under the conditions used, chloramine T has been shown to exclusively oxidize cysteines to cystine and methionine to its sulfoxide[11,12]. Fig. 1 also shows that Peak A retains its identity in RP-HPLC after the DTT reduction of (S^{58}-S^{105}) cystine to (SH^{58},SH^{105}) cysteines[9]. Formation of methionine sulfoxide in other proteins has been shown to produce similar retention shifts in RP-HPLC[13]. This suggests that Peak A contains one or more methionine sulfoxide residues. To test this hypothesis and to elucidate the positions of protein modification, an HPLC peptide map of a tryptic digest has been combined with chemical modification of IL-2. In addition, N-terminal sequencing of a cyanogen bromide digest of IL-2 was performed.

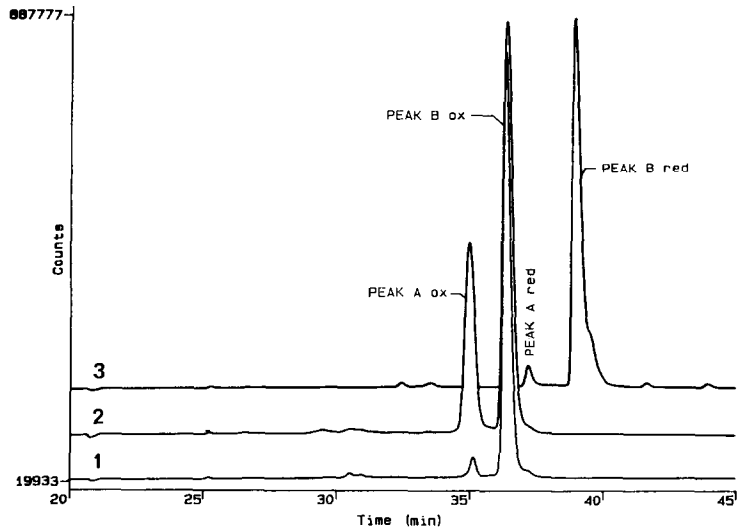

Fig. 1. RP-HPLC of Peaks A and B in desAla^{1}Ser^{125} IL-2. (1) Disulfide-containing (S^{58}-S^{105}) IL-2, showing the location of Peak A and Peak B. (2) A 2-min equimolar chloramine T oxidation of the IL-2 shown in Fig. 1.1 rapidly converts Peak B into Peak A. (3) Cystine (S^{58}-S^{105}) to cysteine (SH^{58},SH^{105}) reduction by DTT of IL-2 from Chromatogram 1, demonstrating the location of reduced Peaks A and B and the integrity of their ratio during reduction. For reduction conditions see Methods (tryptic digestion); Gradient 4 was used; for other conditions see Experimental.

Tryptic digestion of IL-2

Fig. 2 shows the amino acid sequence of IL-2 in which the location of the tryptic and cyanogen bromide cleavage as well as the mutations and deletions made are shown. The experiments establishing the identity of Peak A through tryptic peptide identification, chemical modification, and cyanogen bromide digestion were performed on the desAla^{1}(S^{58}-S^{105})Ser^{125} IL-2 mutein, a protein which can only form one cystine disulfide. Tryptic cleavage was not observed after every lysine and arginine residue, but it was highly reproducible. The identities of the peptides shown in Fig. 3 were established by determinations of amino acid compositions and peptide sequences. In addition, their relative retentions, predicted from the amino acid composition, frequently followed the rule of Browne[14], thus facilitating identification. Peptides corresponding to the entire sequence of IL-2 except T1 are shown. Peptide T1 is hydrophilic and elutes in the void volume of Fig. 3 but has been identified as an early eluting peak in Gradient 1 (not shown). Because of the large amounts of trypsin used, several of the peaks represent trypsin fragments resulting from autodigestion. Trypsin fragments are eluted with the IL-2 peptide T5 and the corresponding sulfoxide, T5 met-S0 (Fig. 3.7).

Cystine-containing material from Peak B was used as the starting material for four different tryptic digestions: (1) the substrate was intact cystine-containing (S^{58}-S^{105}) protein (Peak B_{ox}); (2) the substrate was produced by reduction of cystine to cysteines, followed by carboxymethylation ($SCH_2CO_2H^{58}$,$SCH_2CO_2H^{105}$), (Peak $B_{red/cm}$); (3) the substrate was produced by conversion of Peak B into Peak A with chloramine T (Peak A_{CT}); (4) the disulfide in Peak A_{CT} was reduced and this was

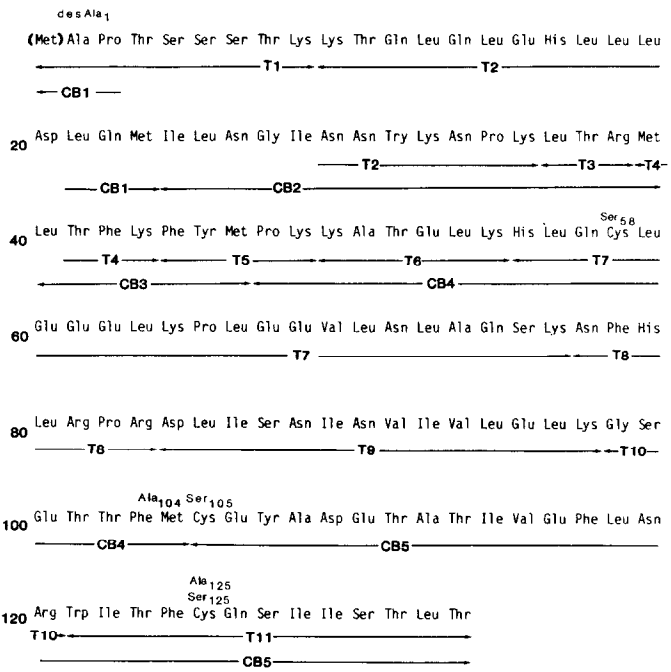

Fig. 2. The recombinant parent amino acid sequence of IL-2, showing the locations of tryptic and cyanogen bromide cleavage. The amino acid residues above the sequence denote the locations and types of site-specific mutations made in constructing the IL-2 muteins.

followed by carboxymethylation (Peak $A_{CT,\ red/cm}$). Fig. 3 shows HPLC maps of these various digests. The peptides that change from one digest to the next are labeled. Peptides common to all four digests are those without cysteine or methionine. These peptides (T1, T3, T8, T9, T11) do not shift in retention time following changes in the oxidation state of the methionine residues or the reduction state of the sulfhydryls and can thus serve as reference peaks in Fig. 3.

The identity of the cystine-linked peptide as well as the methionine sulfoxide peptides can be deduced from the chemical modifications of the intact IL-2. DTT reduction and iodoacetate carboxymethylation of the disulfide-containing peptide (T7 + T10) results in two smaller, more hydrophilic peptides ($T7_{/cm}$ and $T10_{/cm}$, Fig. 3.2). When IL-2 is oxidized with chloramine T to form Peak A_{CT}, the peptides containing methionine shift to shorter retention times, reflecting the increased polarity of the methionine sulfoxide formed ($T2_{met\text{-}SO}$, $T4_{met\text{-}SO}$, $T5_{met\text{-}SO}$, T7 + $T10_{met\text{-}SO}$, Fig. 3.3). If the chloramine T-oxidized IL-2 is reduced and carboxymethylated prior to digestion (Peak $A_{CT,red/cm}$), the T10 peptide has a shorter retention, reflecting methionine sulfoxide oxidation ($T10_{red/cm,\ met\text{-}SO}$, Fig. 3.4).

Authentic Peak A material was purified by RP-HPLC and subjected to tryptic digestion and mapping (Fig. 3.5 and 3.6). The digests of Peak A were indistinguishable from their disulfide-containing or reduced and carboxymethylated Peak B counterparts, except for peptides containing T10. The chromatogram of Peak A_{ox} (Fig. 3.5) does not contain $T4_{met\text{-}SO}$, $T5_{met\text{-}SO}$, or the $T2_{met\text{-}SO}$ cluster, but rather their non-

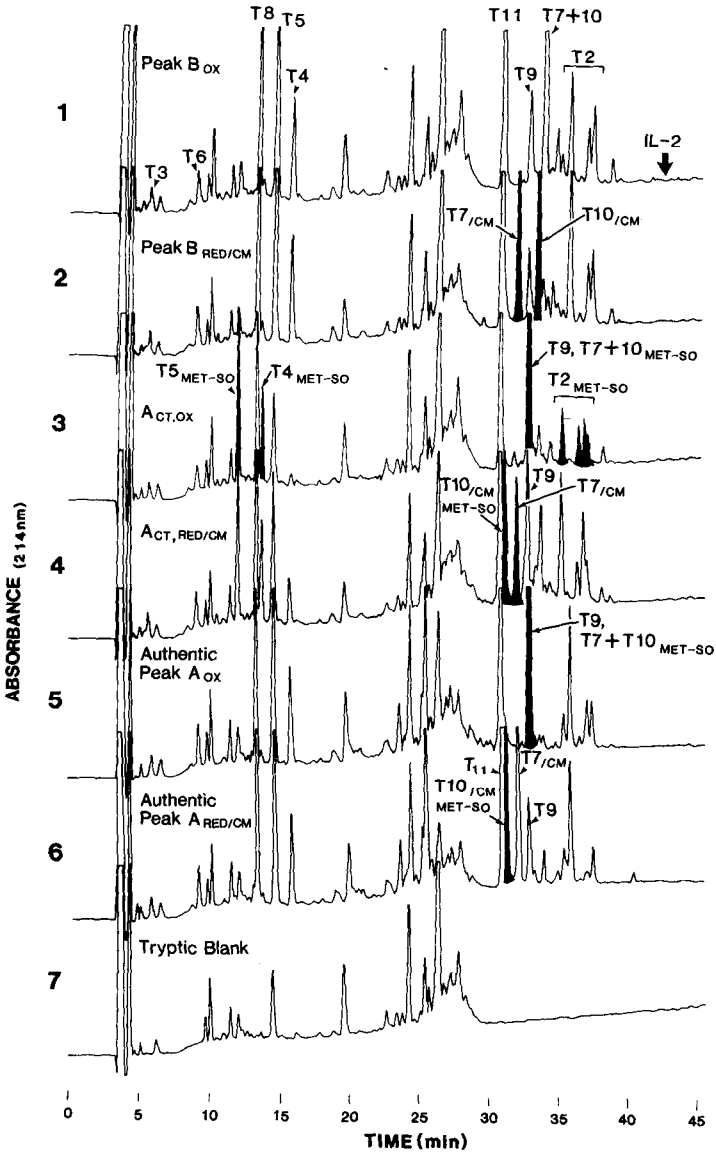

Fig. 3. Tryptic digests of chemically modified desAla^{1}Ser^{125} IL-2. (1) Cystine-containing (S^{58}-S^{105}) Peak B_{ox}; (2) reduced and carboxymethylated Peak B_{ox} to give Peak $B_{red/cm}$ ($S^{58}CH_2CO_2H$, $S^{105}CH_2CO_2H$); (3) conversion of Peak B_{ox} into Peak A_{CT} with chloramine T to generate methionine sulfoxides; (4) reduced and carboxymethylated Peak A_{CT} to give Peak $A_{CT,red/cm}$; (5) authentic cystine-containing (S^{58}-S^{105}) Peak A; (6) reduced and carboxymethylated authentic Peak A to give authentic Peak $A_{red/cm}$ ($S^{58}CH_2CO_2H$, $S^{105}CH_2CO_2H$); (7) a tryptic digest blank showing interfering peptides caused by autodigestion. The shaded peaks represent peptides which change from digest to digest (see text). Gradient 2 was used; for other conditions see Experimental.

oxidized counterparts. However, the T7 + $T10_{met\text{-}SO}$ disulfide-linked peptide is seen to be eluted with T9, while the non-oxidized T7 + T10 disulfide-linked peptide is absent. The Peak $A_{red/cm}$ digest (Fig. 3.6), indicates that methionine oxidation occurs solely at position 104.

Cyanogen bromide digest

A cyanogen bromide digest was also useful for locating the position of methionine oxidation in the IL-2 protein. Under the conditions employed, cyanogen bromide cleaves only peptide bonds on the C-terminal side of unmodified methionyl residues[15]. When a mixture of peptides is sequenced, several PTH amino acid derivatives are observed in each analytical cycle. Since the protein sequence of IL-2 is known, this combination of amino acids can be predicted, and a missing peptide would be noticeable by the absence of amino acids corresponding to its sequence. The differences between the N-terminal sequence and the sequences following the four methionines in IL-2 allow concurrent identification of this combination of peptides.

Both purified authentic Peak A IL-2 and Peak B IL-2 material were digested by cyanogen bromide and the resulting peptide mixtures were sequenced in separate experiments without further fractionation. Eighteen cycles of sequence analysis show that all five peptides in the Peak B cyanogen bromide digest were present in similar amounts, implying that the four methionyl residues in Peak B material were unmodified. In contrast, during the twenty sequencing cycles of the authentic Peak A digest, no PTH amino acid unique to CB5 was recovered in any sequencing cycle, while the PTH amino acids unique to the other four sequences were present at levels comparable with those in the Peak B digest. Thus, in Peak A only the methionine at position 104 failed to be cleaved, indicating a modification solely at that methionine. Although lack of cyanogen bromide cleavage does not indicate the nature of modification, this experiment corroborates the location of the methionyl modification predicted from tryptic mapping.

The combination of tryptic and cyanogen bromide digests provides evidence that methionine 104 can be readily oxidized to its corresponding sulfoxide, resulting in an easily identifiable hydrophilic shift in RP-HPLC retention (Peak A). Peak A_{CT}, which contains significant amounts of methionine sulfoxide at all four methionine positions, displays an additional hydrophilic shift in RP-HPLC retention when compared with authentic Peak A material, containing only one methionine sulfoxide residue. However, extensive methionine oxidation results in a modest decrease in RP-HPLC retention, compared with authentic Peak A IL-2 (Fig. 4). These results imply that methionine 104 is on the surface of IL-2, because not only is it the methionine most readily oxidized but also it has the largest effect upon RP-HPLC retention.

IL-2 muteins

The amino acid sequence of the native IL-2 is shown in Fig. 2. The N-terminal methionyl residue and leader sequence are enzymatically removed in the endogenous processing of the native molecule, but when expressed in *E. coli*, substantial heterogeneity at the N-terminus occurs as a result of incomplete post-translational processing[2]. It has been found that the N-terminal alanine can be removed through

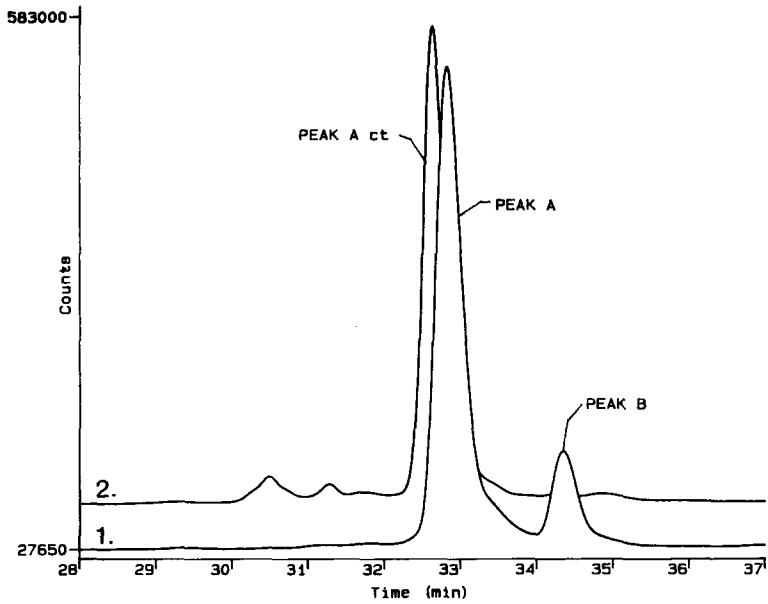

Fig. 4. (1) Chromatogram of authentic Peak A (methionine sulfoxide 104); (2) chromatogram of chloramine T-generated Peak A_{CT} (oxidation at four methionines). Gradient 4 was used; for conditions see Experimental.

genetic engineering, resulting in a homogeneous N-terminal sequence, beginning with proline[4–7]. Only two of the three cysteines in IL-2 are necessary for disulfide bridging in the oxidized protein. Thus, the cysteines were replaced, one at a time, by serine in order to determine the configuration of the native molecule. The desAla^1(S^{58}-S^{105})Ser^{125} IL-2 mutein maintains biological activity equivalent to the native molecule, whereas the corresponding desAla$^1Ser^{58}$(S^{105}-S^{125}) IL-2 mutein and desAla^1(S^{58}-S^{125})Ser^{105} IL-2 analogues are much less active[3,4,6]. This mutation of cysteine to serine at position 125 has the potential advantage of enhanced stability

TABLE I

RETENTION TIMES OF IL-2 MUTEINS IN RP-HPLC

Gradient 3; for other chromatographic conditions, see Experimental.

Mutein	*Retention time (min)*			
	Peak B_{red}	*Peak A_{red}*	*Peak B_{ox}*	*Peak A_{ox}*
Ala^1Cys^{125} (parent)	39.0	36.3	35.0	33.2
Ala^1Ser^{125}	34.6	31.9	31.4	29.6
desAla$^1Cys^{125}$	38.8	36.1	34.9	33.0
desAla$^1Ser^{125}$	34.6	32.1	31.4	29.7
desAla$^1Ala^{125}$	40.9	38.1	36.6	34.6
desAla$^1Ala^{104}Ser^{125}$	32.8	–	30.0	–
desAla$^1Ser^{58}$	38.9	36.4	19.9	–
desAla$^1Ser^{105}$	38.0	35.2	20.3	–

over the native $Cys^{58,105,125}$-containing protein in that disulfide interchange, catalyzed by free cysteine, is minimized. Serine represents a single-atom structural pertubation from cysteine, but other IL-2 muteins, such as the $desAla^1Ala^{125}$ have been constructed to test tertiary conformational integrity. Based upon the structural elucidation of Peak A, substitution of alanine for methionine at position 104 has been made to prevent facile formation of methionine sulfoxide[5,9].

With one exception, the serine mutein which contains no cysteine, all of the IL-2 muteins can undergo intramolecular oxidization of their cysteines to cystine. Native IL-2 contains a disulfide bridge between the cysteine residues at positions 58 and 105 (S^{58}-S^{105})[3]. Oxidation of the parent recombinant molecule $Ala^1(SH^{58}, SH^{105}, SH^{125})$ IL-2 preferentially forms the same cystine (S^{58}-S^{105}) disulfide, which retains full biological activity[6]. Table I shows that when cysteines are oxidized to cystine in the IL-2 muteins, Peak B retention consistently decreases. Non-reducing gel electrophoresis show these intramolecular cystine-containing muteins to be monomeric. Two muteins, $desAla^1Ser^{58}(S^{105}$-$S^{125})$ IL-2 and $desAla^1(S^{58}$-$S^{125})Ser^{105}$ IL-2 produce unusually large hydrophilic retention shifts, 19.1 and 17.7 min respectively, when oxidized. These IL-2 muteins cannot form the normal (S^{58}-S^{105}) disulfide and thus appear to significantly distort their tertiary conformation to form these unnatural disulfides (S^{58}-S^{125} and S^{105}-S^{125}) (Fig. 5). The relative absence of biological activity in these muteins also suggests significant conformational changes[4]. These results imply that unusual RP-HPLC retention and reduced biological activity are correlated through distortions in the protein structure.

Following extended storage, many of the IL-2 mutein preparations contain low levels of Peak A material, which is presumed to represent a modification of methionine 104 to its corresponding sulfoxide (Table I). In addition, the fact that corresponding disulfide-containing and sulfhydryl-containing IL-2 muteins maintain a constant Peak A–Peak B retention interval supports this assumption. The exceptions are the oxidized and reduced $desAla^1Ala^{104}Ser^{125}$ IL-2, oxidized $desAla^1Ser^{58}(S^{105}$-$S^{125})$ IL-2, and oxidized $desAla^1(S^{58}$-$S^{125})Ser^{105}$ IL-2 muteins. The Ala^{104} mutein has been genetically engineered to resist methionine oxidation in either the reduced or oxidized form and contains no Peak A material[5,9]. The $desAla^1Ser^{58}(S^{105}$-$S^{125})$ IL-2 and $desAla^1(S^{58}$-$S^{125})Ser^{105}$ IL-2 muteins contain the unnatural cystine disulfide, and their conformation is distorted relative to the other disulfide-containing IL-2 muteins[4]. It is assumed that methionine 104 no longer oc-

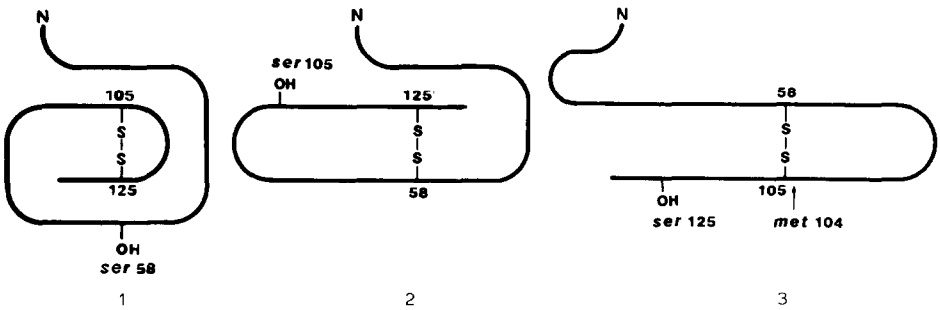

Fig. 5. A simplified schematic diagram of disulfide-oxidized IL-2 mutein structures: (1) $desAla^1Ser^{58}(S^{105}$-$S^{125})$ IL-2; (2) $desAla^1(S^{58}$-$S^{125})Ser^{105}$ IL-2; (3) $desAla^1(S^{58}$-$S^{105})Ser^{125}$ IL-2.

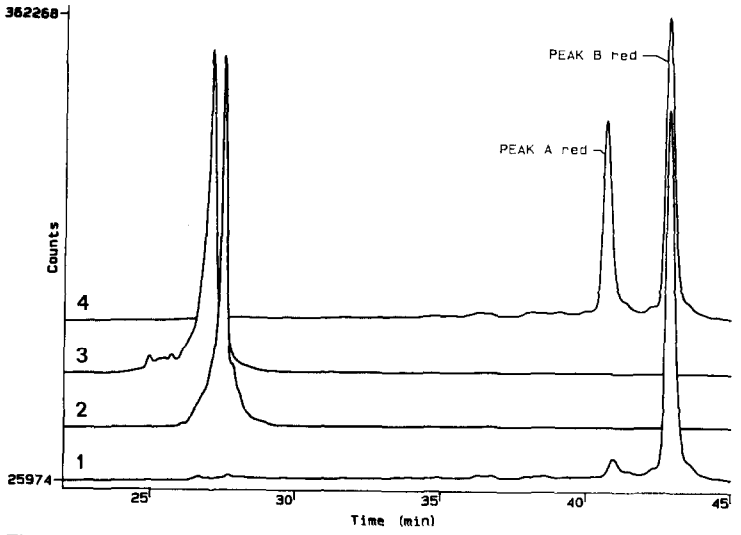

Fig. 6. Oxidation of cysteines and methionines in desAla[1]Ser[58] IL-2. (1) Reduced desAla[1]Ser[58](SH[105], SH[125]), containing 6% Peak A; (2) a chromatogram of the desAla[1]Ser[58](SH[105], SH[125]) IL-2 shown in Fig. 6.1, which has been oxidized to desAla[1]Ser[58](S[105]-S[125]) IL-2; (3) a chromatogram of desAla[1]Ser[58](S[105]-S[125]) IL-2 shown in Fig. 6.2, which has been oxidized with chloramine T to generate methionine sulfoxide; (4) a chromatogram of the IL-2 shown in Fig. 6.3, which has been reduced with DTT to yield desAla[1]Ser[58](SH[105], SH[125]) IL-2 containing 38% Peak A. Oxidation and reduction conditions as in Fig. 1; Gradient 4 was used.

cupies the same exterior position, and thus, the methionine sulfoxide 104 contribution to RP-HPLC retention is very different. Peak A is present in the desAla[1]Ser[58](SH[105],SH[125]) IL-2 and desAla[1](SH[58],SH[125])Ser[105] IL-2 muteins, but Peak A is not identifiable in the corresponding chromatograms of desAla[1]Ser[58](S[105]-S[125]) IL-2 and desAla[1](S[58]-S[125])Ser[105] IL-2. Figs. 6.1 and 6.2 indicate that disulfide bond formation does not produce Peak A in the desAla[1]Ser[58](S[105]-S[125]) IL-2 mutein, implying a different position of the methionine 104 residue, even though Peak A is observed in the cysteine (SH[105], SH[125]) form. Analogous to results in Fig. 4, chloramine T treatment of desAla[1]Ser[58](S[105]-S[125]) IL-2 produces only a small hydrophilic shift in retention (Fig. 6.3), which can be verified as due to methionine sulfoxide formation by the large amount of Peak A material obseved (38%) after subsequent DTT reduction (Fig. 6.4).

We have presented several examples of RP-HPLC retention and chemical reactivity that indicate methionine 104 to be on the protein surface. Molecules containing methionine sulfoxide 104 show a consistent hydrophilic retention shift relative to their non-oxidized methionine 104 counterparts. In cystine-containing variants of desAla[1]Ser[58](S[105]-S[125]) IL-2 and desAla[1](S[58]-S[125])Ser[105] IL-2, containing the unnatural disulfide conformations, a peak with a retention corresponding to oxidized Peak A is not observed. Methionine 104 is the first methionine residue to be oxidized by chloramine T, suggesting its accessibility. In IL-2 where combinations of all four methionines are oxidized with chloramine T, the RP-HPLC retention is only marginally shifted compared with that resulting from methionine oxidation at position 104 alone.

Amino acid deletions at the N-terminal end of IL-2 only marginally affect RP-HPLC retention. Both desAla^{1}Ser^{125} IL-2 and the desAla^{1}Cys^{125} IL-2 muteins differ from Ala^{1}Ser^{125} IL-2 and Ala^{1}Cys^{125} IL-2 muteins in lacking the hydrophobic N-terminal methionine and alanine residues, yet their RP-HPLC retention is not altered (Table I)[2]. By contrast, substitution of cysteine at position 125 with serine or alanine (one-atom substitutions or deletions) yielded significant differences in retention. Previous investigators have found similar types and levels of biological activity in the Jurkat native IL-2, the Ala^{1}Ser^{125}, and the desAla^{1}Ser^{125} IL-2 muteins. This implies that the N-terminal residues do not contribute to the tertiary conformation required for receptor binding[6,7]. The biological activity and RP-HPLC retention results suggest that minor N-terminal amino acid deletions do not alter the tertiary structure of IL-2. One possible explanation is that the N-terminal peptide (T1) is very hydrophilic and constitutes a hydrophilic tail, distal from the hydrophobic face of the protein which binds to the stationary phase in RP-HPLC and, potentially, to the IL-2 receptor. Thus, changes made to the end of this hydrophilic tail may not interfere significantly with the conformation of hydrophobic portions of the IL-2 protein.

The IL-2 muteins listed in Table I contain subtle changes in the primary and secondary protein structure that lead to significant changes in RP-HPLC retention. In some instances, single amino acid changes produce minor changes in retention, while other modifications, such as disulfide formation, can cause large changes in protein conformation and RP-HPLC retention. A detailed quantitative analysis of the RP-HPLC retention of the IL-2 muteins listed in Table I has shown variations in isocratic (55% organic) log K' values of *ca.* 10 000 fold[16]. We believe that the hydrophobic nature of the IL-2 protein is responsible for the observed chromatographic behavior, and that substantial tertiary protein structure (folding) exists while IL-2 is on the surface of the stationary phase. Proteins normally denature and unfold under these RP-HPLC conditions[17]. Thus, RP-HPLC retention gives structural clues consistent with observations of the chemical and biological activity of the IL-2 muteins discussed.

REFERENCES

1 L. E. Henderson, J. F. Hewetson, R. F. Hopkins, R. C. Sowder, R. H. Neubauer and H. Rabin, *J. Immunol.*, 131 (1983) 810.
2 H. W. Lahm and S. Stein, *J. Chromatogr.*, 326 (1985) 357.
3 R. J. Robb, R. M. Kutny, M. Panico, H. R. Morris and V. Chowdhry, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 6486.
4 A. Wang, S. D. Lu and D. Mark, *Science*, 224 (1984) 1431.
5 R. Halenbeck, S. D. Lu and K. Koths, *Fourth Annual Stony Brook Symposium, Protein Engineering in Basic Science, Industry, and Medicine, May 20–22, 1985*, Abstract 16.
6 M. Doyle, M. T. Lee and S. Fong, *J. Biol. Resp. Modif.*, 4 (1985) 96.
7 S. A. Rosenberg, E. A. Grimm, M. McGrogan, M. Doyle, E. Kawasaki, K. Koths and D. Mark, *Science*, 223 (1984) 1412.
8 Z. Shaked and S. Wolfe, *U.S. Pat.*, 4 450 787 (1985); assigned to Cetus Corp.
9 K. Koths, R. Halenbeck, A. Wang, P. McCabe, A. Boosman and M. Doyle, in preparation.
10 R. L. Sokoloff and R. P. Frigon, *Anal. Biochem.*, 118 (1981) 138.
11 N. Brot and H. Weissbach, *Trends Biochem. Sci.*, 7 (1982) 137.
12 N. Brot and H. Weissbach, *Arch. Biochem. Biophys.*, 223 (1983) 271.

13 A. L. Frelinger III and J. E. Zull, *J. Biol. Chem.*, 259 (1984) 5507.
14 C. A. Browne, H. P. J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
15 E. Gross and B. Witkop, *J. Biol. Chem.*, 273 (1961) 1856.
16 M. G. Kunitani, D. J. Johnson and L. R. Snyder, presented at the *Tenth International Symposium on Column Liquid Chromatography, San Francisco,CA, May 18–23, 1986.*
17 R. H. Ingraham, S. Y. M. Lau, A. K. Taneja and R. S. Hodges, *J. Chromatogr.*, 327 (1985) 77.