Role of Single Disulfide in Recombinant Human Tumor Necrosis Factor-α*

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Two analogs of tumor necrosis factor-α (TNF-α) were produced by in vitro site-directed mutagenesis. In these analogs, cysteine residues at positions 69 and 101, which form a disulfide bond, were changed to alanine or leucine. CD spectra showed that the analogs are apparently similar in secondary and tertiary structure to the natural sequence TNF-α. In addition, the molecular size of the analogs was identical to that of the natural sequence TNF-α as determined by gel filtration. However, fluorescence spectra and quenching indicated that the removal of the disulfide bond alters the local conformation around tryptophan residues.

The cytolytic, macrophage activation, and lipogenic activities decreased in the order of the natural sequence TNF-α > the alanine analog > the leucine analog, suggesting that the surface involving the disulfide bond plays a role in these biological functions and the introduced modifications decrease the activity. Differential effect of the modifications was suggested in the antiviral activity, since in this assay only the leucine analog showed significantly lower activity.

Human tumor necrosis factor (TNF-α) has 2 cysteine residues at positions 69 and 101 and these residues form an intramolecular disulfide bond (Davis et al., 1987). It has been shown that TNF-α is a trimer (Arakawa and Yphantis, 1987) and the subunit interactions occur by means other than disulfide bonds (Davis et al., 1987; Aggarwal et al., 1985; Shirai et al., 1985). Conformational analyses by CD and secondary structure prediction showed that the disulfide bond is located at the protein surface and its reduction has no detectable effect on the secondary and tertiary structures of the protein (Davis et al., 1987). It is therefore of interest to examine whether the disulfide bond plays any role in the local structure and properties involved in the biological activity. In the previous study, we have synthesized a gene coding for TNF-α, expressed the corresponding protein in Escherichia coli and purified it by a series of chromatographic procedures (Davis et al., 1987). The purified protein showed a cytolytic activity identical to that for the natural protein preparation, and hence was used for structural characterization. In this study, cysteines 69 and 101 were changed to either alanine or leucine, and these analogs were designated as [Ala]TNF-α and [Leu]TNF-α. This paper describes the conformational properties and biological activities of these analogs in comparison with the natural sequence protein.

Recent studies on recombinant TNF-α revealed that the protein displays a number of biological functions such as antiviral (Wong and Goeddel, 1986; Mestan et al., 1986; Arakawa et al., 1987) and cachectic (Beutler and Cerami, 1986) as well as cytolytic activities (Sugarman et al., 1985; Wang et al., 1985). In view of the similarity between TNF-α and TNF-β (Pennica et al., 1985; Aggarwal et al., 1985), it was expected that TNF-α would exhibit macrophage activation activity as does TNF-β. Since these different activities may involve different domains of the molecule, the analogs may exhibit differential activity in these assays when compared with the natural sequence TNF-α.

MATERIALS AND METHODS

TNF-α analogs were created by in vitro site-directed mutagenesis, making amino acid substitutions at positions 69 and 101, from cysteine to alanine or leucine. Mutagenic reactions were performed in M13 vectors using synthetic oligonucleotides as mutagenic primers for in vitro DNA synthesis. Clones containing the mutant analog gene were verified by DNA sequence analysis and then subcloned into an expression vector. For comparative purposes, the same expression system was used for TNF-α and the analogs. Derivatives of E. coli strain C600 harboring these plasmids were induced to express high levels of these proteins using standard fermentation procedures.

The analogs were purified essentially by the same procedures as described for the natural sequence protein except for the removal of dithiothreitol throughout the purification (Davis et al., 1987). Briefly, inclusion bodies, obtained after disruption of E. coli cells, were solubilized in the presence of urea (ultrapure urea) and subjected to cation exchange chromatography at pH 4.5 and then anion exchange column chromatography at pH 9.0, both in the presence of urea. The purified protein in urea was renatured by a 10-fold dilution into 20 mM Tris-HCl without urea (pH 8.5 at room temperature) maintained at 5 ± 3°C, followed by anion exchange chromatography in 20 mM Tris, pH 8.5, in the absence of urea. The bound protein was eluted with 0.1 M NaCl in the same buffer, and stored at 4°C in 20 mM Tris, pH 8.2, 0.1 M NaCl.

The cytolytic activity was assayed using mouse L929 cells in the absence of actinomycin D, as described previously (Arakawa et al., 1987). The antiviral activity was assayed in euephalomyocarditis virus-infected HeLa cells. The samples to be compared were assayed at the same time.

In vitro effects of TNF-α on lipogenesis in mouse adipocytes were observed as previously described (Torti et al., 1985). Briefly, 3T3-L1 or TA1 mouse adipocyte cell lines were plated and grown in Eagle's basal medium with 10% fetal calf serum. Dexamethasone (10−7 M), human insulin (5 μg/ml), and isobutylmethylxanthine (0.5 mM) were added to the medium when the cells reached confluency to speed differentiation and enhance the production of intracellular lipid. TNF-α and the analogs were also added and their effects on the production of lipid were compared to samples without TNF-α. Observations were made 6 days after the addition of hormones and TNF-α. Intracellular lipid inclusions were clearly identified by staining with oil red O.

Macrophage activation was measured in terms of enhanced hydro-

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1 The abbreviation used is: TNF, tumor necrosis factor.
gen peroxide release from the human myeloid cell line U937 as previously described (Andrew et al., 1984). Briefly, aliquots of 1 x 10^6 U937 cells grown in RPMI 1640 medium containing 10% fetal calf serum were dispensed into 12 x 75-mm plastic tubes. Dilutions of TNF-α and the analogs, with or without IFN-γ (1000 units/ml), were added to the tubes which were then incubated for 3 days at 37°C. Hydrogen peroxide release in response to a 1-h incubation with phorbol myristate acetate (5 μg/ml) was then estimated by the oxidation of phenol red catalyzed by horseradish peroxidase.

Circular dichroic spectra were determined on a Jasco J-500C spectropolarimeter at room temperature equipped with an Oki 800 model 30 computer. The data were expressed as mean residue ellipticity calculated using a mean residue weight of 111. Fluorescence spectra were determined on a Perkin-Elmer LS-5 luminescence spectrophotometer at room temperature.

Fluorescence quenching experiments were carried out using acrylamide of electrophoresis purity from Bio-Rad, KI from Sigma, and CsCl of optical grade from Gallard-Schlesinger as a quencher. Quenching experiments were carried out using a 0.5-cm cell thermostatted at 21°C. Small volumes of a concentrated stock solution of acrylamide were added to TNF-α solutions. Excitation was at 300 nm to minimize acrylamide absorbance. For KI and CsCl quenching experiments, separate samples were prepared for each quencher concentration. A 4 M NaCl and 4 M solution of theionic quencher were added so that the ionic strength of each sample was the same. The KI and CsCl quenching experiments were performed with excitation wavelength at 300 and 280 nm, respectively. Corrections were made for dilution and for absorbance by the quencher: 

\[ F_0 = F \cdot \left(1 - \frac{1}{1 + \left(\frac{I}{I_0}\right)^n}\right) \]

where

\[ I \]

is the change in the absorbance by addition of the quencher (Lehrer and Leavis, 1978).

Protein concentrations were spectrophotometrically determined using the extinction coefficient of 1.62 at 280 nm for a 0.1% TNF-α solution (Davis et al., 1987). The same value was used for TNF-α and the two analogs.

**RESULTS**

Secondary structure prediction according to the method of Chou and Fasman (1978) indicated that the tetrapeptide of Cys⁶⁶ to Thr⁷² and Ser⁹⁹ to Gin¹⁰² have an overall β-turn potential of 4.43 x 10⁻⁴ and 4.14 x 10⁻⁴, respectively. These values are about 8-fold higher than the average value obtained by Chou and Fasman (1978), and suggest that Cys⁹⁹ and Cys¹⁰¹, which form a disulfide bond, are at the first and third positions of β-turns, respectively. Alanine was chosen for substitution because of similar side chain volume (52.6 Ǻ³) and hydrophobicity (750 cal/mol) to half-cystine (68.3 Ǻ³ and 1000 cal/mol) according to Bigelow (1967). Leucine was chosen to increase the hydrophobicity of the side chain (2400 cal/mol). Changing cysteine to alanine or leucine decreases the β-turn potential of the Cys⁶⁶ to Thr⁷² sequence by approximately 60% and that of the Ser⁹⁹ to Gin¹⁰² sequence by approximately 70%. However, the resultant β-turn potentials after the amino acid substitutions are still 2- to 3-fold higher than the average value, suggesting that these tetrapeptides form β-turns (Chou and Fasman, 1978). Therefore, the alterations in conformation and activity upon mutation may be analyzed in terms of the effect of mutation on the local environment about the disulfide bond in the natural sequence TNF-α.

Structure of [Ala]TNF-α and [Leu]TNF-α—CD spectra of TNF-α and the analogs are shown in Fig. 1. Far UV spectra, shown in Fig. 1A, show a maximum at 200.5 nm and a minimum around 219 nm, essentially identical to those observed for the natural sequence TNF-α. However, the CD intensities at 219 nm appear to be slightly lower for the two analogs than for the natural sequence TNF-α, suggesting slight effects of the mutations on the secondary structures of the protein, the effect being similar for alanine and leucine substitutions. In the previous paper (Davis et al., 1987), the far UV CD of the natural sequence TNF-α was interpreted in terms of an essentially non-helical, β-sheet-rich structure.

The results indicate apparently no effects of the substitutions on the overall secondary structure.

The near UV CD spectra are also similar for the natural sequence TNF-α and the analogs, indicating that the mutations did not cause any drastic changes in tertiary structure of the protein. However, small differences appear to be present, suggesting a small perturbation of tertiary structure, or local conformation by the mutations.

Fluorescence spectra of the protein when excited at 280 nm are shown in Fig. 2. The spectrum for the natural sequence TNF-α showed a broad peak maximum from 319 to 331 nm. This may reflect two tryptophans in TNF-α, which are in different environments. These peak wavelengths suggest that the tryptophans are largely buried in the interior of the protein molecule. The fluorescence spectra for the 2 analogs are similar, characterized by a peak around 331 nm. Comparison of these with the spectrum for the natural sequence TNF-α shows two distinct differences: peak positions and intensity. The observed peak at 319 nm in the natural sequence TNF-α appears as a shoulder of the 331-nm peak in the analogs. The fluorescence intensity at maximum increased by 1.8-fold in the analogs relative to the intensity for the natural sequence TNF-α. It seems, therefore, that in these analogs, the fluorescence intensity of the 331-nm peak is essentially the same in the 2 analogs, with 300 nm excitation, indicating that the observed differences are due to alteration in tryptophan environments.

Fig. 3 shows the fluorescence quenching data with KI (A) and acrylamide (B). The two analogs showed a similar quenching pattern using KI as a quencher, indicating an accessibility of the tryptophans to this quenching agent. The natural sequence TNF-α showed little quenching by KI (solid square), however, indicating that the tryptophans are not accessible within a measurable range. In the case of CsCl as a quencher, there was no detectable quenching for all the three proteins, indicating that the tryptophans in the analogs, which can be quenched by KI, are not accessible to the positive ion quencher, Cs⁺. The results with acrylamide are obtained for the natural sequence TNF-α and [Ala]TNF-α. In this case, both the natural sequence TNF-α and the analog showed...
weak quenching to the same extent, within experimental error. Therefore, the tryptophans in both TNF-α molecules are accessible to the non-ionic quencher, acrylamide, and the accessibility is the same for the two proteins.

Gel filtration of the three proteins showed an essentially identical elution position, at the elution position close to that for ovalbumin. This suggests that the amino acid substitution did not effect oligomerization of the protein.

Biological Activity—Cytolytic activity of the proteins was assessed on mouse L929 cells. All three proteins showed a linear dose dependence of the activity (data not shown). The activity was distinctly different between the proteins. [Ala]TNF-α and [Leu]TNF-α showed about 15-fold and 5-fold lower activity, respectively, than the natural sequence TNF-α, which showed \(-1 \times 10^7\) units/mg. The same order of the activity was also observed in lipogenic and macrophage activation assays, as described below.

The antiviral activity on HeLa cells was compared for the three proteins and for recombinant E. coli-derived IFN-γ. The observed results were: IFN-γ, \(1.2 \times 10^6\) units/mg; the natural sequence TNF-α, \(6 \times 10^6\) units/mg; [Ala]TNF-α, \(5 \times 10^6\) units/mg; [Leu]TNF-α, \(1 \times 10^6\) units/mg. Thus, only [Leu] TNF-α showed significantly lower activity than the natural sequence TNF-α.

Macrophage activation activities of IFN-γ alone and in combination with the three forms of TNF-α analyzed in this study were compared. It has been shown that IFN-γ alone is suboptimal in activating macrophages and may act as trigger in a two-step activation process (Krammer et al., 1985; Pace et al., 1983). We therefore assayed TNF-α in the presence and absence of IFN-γ. TNF-α alone had no significant macrophage activation activity, while 1 ng/ml TNF-α assayed in the presence of 1000 units/ml IFN-γ gave a 4- to 5-fold increase in the amount of H₂O₂ released. Molar quantities of H₂O₂ released were as follows: IFN-γ alone, 4.36 ± 0.76 μM; natural sequence TNF-α plus IFN-γ, 20.31 ± 0.94 μM; [Ala]TNF-α plus IFN-γ, 15.86 ± 1.87 μM; [Leu]TNF-α plus IFN-γ, 10.31 ± 0.63 μM.

The effect of TNF-α on the production of intracellular lipid was observed microscopically on oil red O-stained 3T3-L1 and TA1 mature mouse adipocyte cultures. Typically, 6 days after the addition of hormones, 80% of the cells would contain large intracellular lipid droplets. The percentage of cells containing lipid decreased when increasing concentrations of TNF-α were added (data not shown). When 0.3 ng/ml natural sequence TNF-α was added to the cultures, only 10% of the cells stained for intracellular lipid while the same concentration of [Ala]TNF-α and [Leu]TNF-α inhibited lipogenesis to a far lesser extent with lipid in 60-70 and 80% of the cells, respectively. It was necessary to add 3 ng/ml [Ala]TNF-α and 30 ng/ml [Leu]TNF-α to see only 10% lipid inclusion.

It is interesting to note that concentrations of TNF-α above 10 ng/ml produced lysis of the mouse adipocytes, and again, as seen with the cytolytic activity on mouse L929 cells, it took similar relative increases in TNF-α analog concentrations to produce the same degree of lysis.

**DISCUSSION**

In the previous paper (Davis et al., 1987), it was shown that the single disulfide in TNF-α plays no detectable role in secondary and tertiary structure of the protein, as determined by near and far UV CD spectra. This was confirmed by the...
observations in this paper that the near and far UV CD spectra are almost identical for the natural sequence TNF-\(\alpha\) and the analogs, which lack cysteine residues and hence the disulfide bond. In addition, the observed oligomerization of the analogs supports the previous conclusion that the oligomer is formed by means other than disulfide bond. However, fluorescence spectra and quenching showed a distinct difference in local conformation around tryptophans. These results suggest that the disulfide bond in the natural sequence TNF-\(\alpha\) is not essential in maintaining the overall conformation, but contributes to the local conformation.

The fluorescence results give some insight into the local conformation of the natural sequence TNF-\(\alpha\) and analogs. The strongly enhanced fluorescence intensity, in particular at 331 nm, by the mutations suggest that the environment for at least one of the tryptophans is altered by the mutations. It seems that the tryptophans are accessible, although partially, only to non-ionic quencher (acrylamide) in the natural sequence TNF-\(\alpha\). The natural sequence TNF-\(\alpha\) showed no quenching by \(I^-\) and \(Cs^+\), suggesting a possibility that the tryptophans are in a hydrophobic environment, which agrees with their blue-shifted fluorescence maxima. The analogs showed quenching by \(I^-\), but not \(Cs^+\) suggesting that the removal of the disulfide bond decreases the hydrophobicity of the tryptophan environment so that \(I^-\) can penetrate into the molecule. The lack of quenching by \(Cs^+\) for the analogs suggests that the tryptophans are also protected from the positive ion by an electrostatic-free energy barrier against this ion.

The results of gel filtration indicated that the three proteins are identical in molecular size in solution. This agrees with the CD results that the overall conformations are the same for these molecules, and suggests that the local conformational difference between the natural sequence TNF-\(\alpha\) and the analogs has no effect on the oligomerization, which suggests that the surface involving the disulfide bond is not at a protein-protein contact point in the oligomer. The observed biological activities for the analogs indicate that the disulfide bond is not an absolute requirement for all the biological activities studied, i.e. the antiviral, cytolytic, lipogenic, and macrophage activation activities. However, the observed significant decreases in these activities except for antiviral suggest that the surface involving the disulfide bond plays a role in these activities. Differential effect of the mutations on the biological activities was suggested in the antiviral assay, which showed only [Leu]TNF-\(\alpha\) significantly different from the natural sequence TNF-\(\alpha\).

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