Formation of an Active Dimer during Storage of Interleukin-1 Receptor Antagonist in Aqueous Solution

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ABSTRACT The degradation products of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) formed during storage at 30°C in aqueous solution were characterized. Cationic exchange chromatography of the stored sample showed two major, new peaks eluting before (P1) and after (L2) the native protein, which were interconvertible. Size-exclusion chromatography and electrophoresis documented that both the P1 and L2 fractions were irreversible dimers, formed by noncovalent interactions. A competition assay with interleukin-1 indicated that on a per monomer basis the P1 and L2 dimers retained about two-thirds of the activity of the native monomer. Infrared and far-UV circular dichroism spectroscopies showed that only minor alterations in secondary structure arose upon the formation of the P1 dimer. However, alteration in the near-UV circular dichroism spectrum suggested the presence of disulfide bonds in the P1 dimer, which are absent in the native protein. Mass spectroscopy and tryptic mapping, before and after carboxymethylation, demonstrated that the P1 dimer contained an intramolecular disulfide bond between Cys-66 and Cys-69. Although conversion of native protein to the P1 dimer was irreversible in buffer alone, the native monomer could be regained by denaturing the P1 dimer with guanidine hydrochloride and renaturing it by dialysis, suggesting that the intramolecular disulfide bond does not interfere with refolding. Analysis of the time course of P1 formation during storage at 30°C indicated that the process followed first-order, and not second-order, kinetics, suggesting that the rate-limiting step was not dimerization. It is proposed that a conformational change in the monomer is the rate-limiting step in the formation of the P1 dimer degradation product. Sucrose stabilized the native monomer against this process. This result can be explained by the general stabilization mechanism for this additive, which is due to its preferential exclusion from the protein surface.

INTRODUCTION

Development of a protein therapeutic agent requires not only production of a sufficient quantity of homogeneous, highly active protein, but also a stable formulation suitable for storage and delivery. Obtaining such formulations is often difficult because of the intrinsic instability of proteins in aqueous solutions, which are often the preferred mode of preparation. One of the most common manifestations of protein damage during storage in aqueous solution is the formation of non-native protein aggregates, which often precipitate (Arakawa et al., 1993). These aggregated molecules are very similar to those formed during short-term thermal unfolding experiments. The unfolded molecules associate into aggregates that characteristically have a high content of non-native intermolecular β sheet structure (Dong et al., 1995).

The first goal of the current study was to characterize the degradation products formed during long-term storage of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) in aqueous solution. This was of interest because rhIL-1ra belongs to a structural class of proteins, including acidic and basic fibroblast growth factors (aFGF and bFGF), keratinocyte growth factor (KGF), and interleukin-1 (IL-1), all of which show extensive precipitation of non-native molecules upon long-term storage in aqueous solution at moderate temperatures; i.e., above room temperature, but lower than the respective proteins' melting temperatures (Gu et al., 1991; Tsai et al., 1993; Kajio et al., 1992; Volkin et al., 1993; Chen et al., 1994). The X-ray crystal structure of IL-1ra (Vigers et al., 1994) shows that it consists of 12 β-strands, which are folded into an overall topology similar to that observed with aFGF, bFGF, and IL-1 (Finzel et al., 1989; Priestle et al., 1989; Graves et al., 1990; Ago et al., 1991; Clore et al., 1991; Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991; Shaanan et al., 1992). The native forms of all of these proteins are monomeric with no tendency to associate reversibly into native dimers and oligomers (Arakawa et al., 1993; Wen et al., 1996; J. Wen, J. S. Philo, and T. Arakawa, unpublished observation; B. Brandhuber, private communication). Based on this background we fully expected to find that rhIL1-ra would also form extensive, non-native protein aggregates during storage. However, as detailed below we were surprised to find that the major degradation product was a dimer that formed irreversibly, but which retained very native-like structure and biological activity.

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This paper is dedicated to Prof. Serge N. Timasheff on the occasion of his 70th birthday.

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The second goal of the current study was to determine whether approaches used to ameliorate aggregation of aFGF, bFGF, KGF, and IL-1 would also attenuate formation of the dimeric species of rhIL-1ra. This is an intriguing question because it has been shown that in cases where non-native aggregates are produced, kinetic stability can be improved with the addition of co-solvents (e.g., polyols, sugars, or polymers) or specific ligands that are known to increase the thermodynamic stability of the native monomer (Gerlsma, 1968, 1970; Frigon and Lee, 1972; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982, 1985; Timasheff, 1992, 1995; Arakawa et al., 1993; Volkin et al., 1993; Chen et al., 1994; Vrkljan et al., 1994; Liu and Bolen, 1995). Aggregation is attenuated because formation of the unfolded form, which serves as the precursor of the non-native aggregates, is thermodynamically less favorable. The same co-solvents that cause this increase in the free energy of denaturation of monomers are also known to foster assembly of polymerization-competent proteins into the higher-order native oligomers (Timasheff, 1992, 1995; Arakawa et al., 1993). Thus, if the formation of the dimeric form of rhIL-1ra is due to self-association of the native monomer during storage, one might expect sucrose to accelerate this process. In contrast we found that, as with proteins that form non-native aggregates, sucrose inhibited formation of the rhIL-1ra dimer. The putative mechanism for this effect, as well as the detailed structural characterization of the dimer, are described below.

MATERIALS AND METHODS

Pharmaceutical-quality rhIL-1ra was produced and purified at Amgen Boulder (formerly Synergen). The protein was greater than 99.5% homogeneous based on size-exclusion chromatography and approximately 98% pure based on cation-exchange chromatography. Protein solutions (100 mg/ml) containing various concentrations of sugar were prepared in 10 mM sodium citrate buffer (pH 6.5 at 23°C) and 140 mM sodium chloride, and stored at 30°C until analysis. Control samples were stored at −70°C.

High performance liquid chromatography

High performance liquid chromatography (HPLC) analyses were carried out on a Beckman HPLC system, which was interfaced with system GOLD (Beckman Instruments Inc., Fullerton, CA) for programming and data acquisition. Cation-exchange HPLC (CEX-HPLC) was carried out using a Biogel® SP-5-PW column (7.5 mm × 7.5 cm, BioRad Laboratories, Hercules, CA), a flow rate of 0.5 ml/min and 20 mM MES (pH 5.5) as a running buffer. After an injection of 500 μg protein, the protein was eluted with a linear 0 to 1 M sodium chloride gradient. Size-exclusion chromatography HPLC (SEC-HPLC) was carried out using a TSK-Gel® G2000 SWXL column (7.8 min × 30 cm, TosoHaas, Montgomeryville, PA), a flow rate of 0.5 ml/min, and 10 mM sodium citrate (pH 6.5) with 140 mM sodium chloride as the running buffer. For each analysis, 500 μg protein was injected.

Potency bioassay

The potency of rhIL-1ra was measured by its ability to inhibit the effect of interleukin-1α (IL-1α). IL-1α is cytotoxic to the human melanoma cell line A375, clone 6–14 (obtained from K. Matsushima, in J.J. Oppenheim's lab, Laboratory of Immunoregulation, Biological Response Modifier Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Frederick Cancer Research Facility, Frederick, MD). rhIL-1ra inhibits IL-1α cytotoxicity in a dose-dependent manner. A375 cells were treated with serial dilutions of rhIL-1ra standard and samples in the presence of 1 ng/ml IL-1α. Cell survival at approximately 72 h was assessed by the addition of a solution of XTT (sodium 3,3′-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) and menadione. Surviving cells metabolize XTT to a water-soluble formazan product, which was quantitated by measurement of optical density (Roehm et al., 1991). Dose-response curves were fit to a weighted four-parameter logistic model, and ED_{50} values were calculated.

Fourier transform infrared (FTIR) spectroscopy

Infrared spectra of aqueous protein solutions were recorded at 23°C with a Nicolet Magna 550 Fourier transform infrared spectrometer (Nicolet Instruments Co., Madison, WI), equipped with a dTGS detector. Protein solutions (54 mg/ml in 10 mM citrate, 140 mM NaCl at pH 6.5) were prepared for infrared measurement in a sample cell (Beckman FH-01) that used CaF_2 windows separated by a 6-mm spacer. For each spectrum, a 256-scan interferogram was collected in single beam mode, with a 4 cm^{-1} resolution. The reference spectra were recorded under identical conditions with only buffer in the cell. The spectra for liquid and gaseous water were subtracted from the protein spectra, as appropriate, according to previously established criteria (Dong et al., 1990; Dong and Caughey, 1994). The final protein spectrum was smoothed with a seven-point function to remove white noise. Second derivative spectra were calculated with the derivative function of Nicolet Omnic software.

Circular dichroism (CD) spectroscopy

CD spectra were determined on a Jasco J-500C spectropolarimeter (Jasco Inc., Easton, MD) using a 1-cm pathlength cuvette for the near-UV and a 0.02 cm pathlength cuvette for the far-UV. The spectra shown are the average of 10 accumulations, and were calculated assuming an $\epsilon_{280}^{0.1\%}$ of 0.77 at 280 nm for the rhIL-1ra solution and a mean residue weight of 112.8. Protein solutions of 1 to 1.4 mg/ml in 10 mM citrate, 140 mM NaCl at pH 6.5 were studied. The secondary structure was estimated based on a previously published computational method (Greenfield and Fasman, 1969).

ANS binding

The 1,8-anilinonaphthalenesulfonate (ANS) binding assay was performed on an SLM Aminco 500 spectrofluorometer, using protein solutions of 0.2 mg/ml, a cuvette with a 0.5-cm pathlength, and slit widths of 5 mm. After first recording the intrinsic fluorescence upon excitation at 280 nm, increasing amounts of ANS were added, and the spectra from 400 to 520 nm recorded upon excitation at 380 nm.

Tryptic mapping and mass spectrometry

Tryptic mapping of rhIL-1ra was initiated by denaturing 1 mg/ml protein with 1.6 M guanidine hydrochloride in the presence 0.7 mM iodoacetic acid. Reducing agent was not used to preserve disulfide bonds during digestion. The denatured protein was digested with trypsin overnight at 37°C. The digested sample was analyzed with reversed-phase HPLC using a Vydac C4 column (4.6 mm × 25 cm, 5 μm particle size). Acetonitrile was used to develop a gradient from 0 to 60%.

Mass spectrometry was carried out using a Sciex triple quadripole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada) calibrated with polypropylene mass standards. Mass determination was performed on samples purified on reverse phase HPLC, which were infused at 10 ml/min and ionized by an orifice potential of 70 V. Sample

ion streams were scanned in the first quadripole from 400 to 2400 atomic mass units (amu). Deconvolution of raw mass data was performed using Mac Biospec software provided by Sciex.

Curve-fitting for the order of reaction

The formation of P1 dimer was monitored with 100 mg/ml rhIL-1ra solution during storage at 30°C, as described above. Samples were withdrawn at various times and analyzed with CEX-HPLC to determine the amount of P1. To calculate the rate of P1 formation, the following general equation (Benson, 1960) was used.

$$\frac{d[\mathrm{P1}]}{dt} = k[C]^n$$

where $d[\mathrm{P1}]/dt$ is the change in the concentration of native rhIL-1ra monomer to form P1 dimer with time (days), $[C]$ is the concentration of native rhIL-1ra monomer, k is the rate constant in appropriate units, and n is the order of the reaction. For a first-order reaction, a semilogarithmic coordinate (log[P1]) is needed to produce a linear plot. In contrast, a plot of 1/[P1] versus time produces a linear relationship for a second-order reaction.

RESULTS AND DISCUSSION

Structure and function of degradation products arising during storage of rhIL-1ra

In contrast to previous results with aFGF, bFGF, KGF, and IL-1, long-term storage (at 30°C) of aqueous solutions of rhIL-1ra resulted only a relatively small production of visible precipitates (approximately <1% of total protein). To determine the other, more dominant, degradation products, the stored sample was filtered with a 0.2-μm membrane to remove precipitated material and analyzed by various chromatographic procedures. Among many techniques tested (data not shown), CEX-HPLC provided the best separation of these products. Fig. 1 shows the CEX-HPLC chromatograms of rhIL-1ra before and after storage for 2 months at 30°C. Various peaks shown were significantly increased during storage. Of the degradation products represented by these peaks, the fraction eluting (designated as P1) just before the native protein was the major species; it constituted 8% of the total peak area, including the native peak. The P1 fraction was collected and analyzed with various biochemical and biophysical techniques to characterize the nature of the modifications that had occurred.

Reinjection of the P1 fraction onto the CEX-HPLC column resulted in two peaks, P1 and L2 (so designated because it was the second fraction eluting after the native protein), indicating that these two forms are isomers (Fig. 1). Reinjection of the L2 also resulted in the appearance of a P1 peak (data not shown). Why these isomeric forms have different retention times in CEX-HPLC is not clear. More importantly, whenever P1 and L2 were reinjected there was not the appearance of the peak for native protein in the chromatogram (Fig. 1 and data not shown). These results document that the formation of the P1 and L2 degradation products is not reversible, under these conditions.

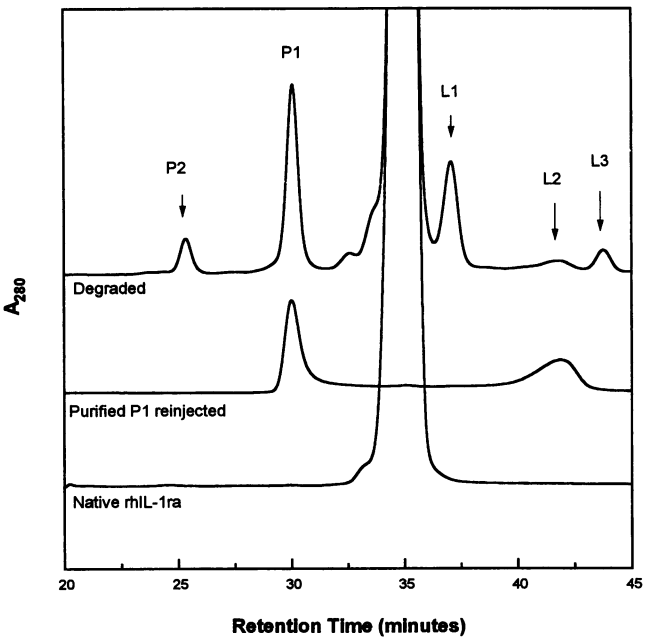

FIGURE 1 CEX-HPLC chromatograms of rhIL-1ra and purified P1 fraction. The experimental sample was incubated for 2 months at 30°C and the control rhIL-1ra sample was stored for 2 months at −70°C.

Reinjection of the P1 fraction onto an SEC-HPLC column demonstrated that P1 was a dimeric form of rhIL-1ra (Fig. 2). The L2 fraction was also the dimeric form of rhIL-1ra (data not shown). Inasmuch as SDS-PAGE showed a monomeric molecular weight (data not shown), these dimers are formed by noncovalent interactions.

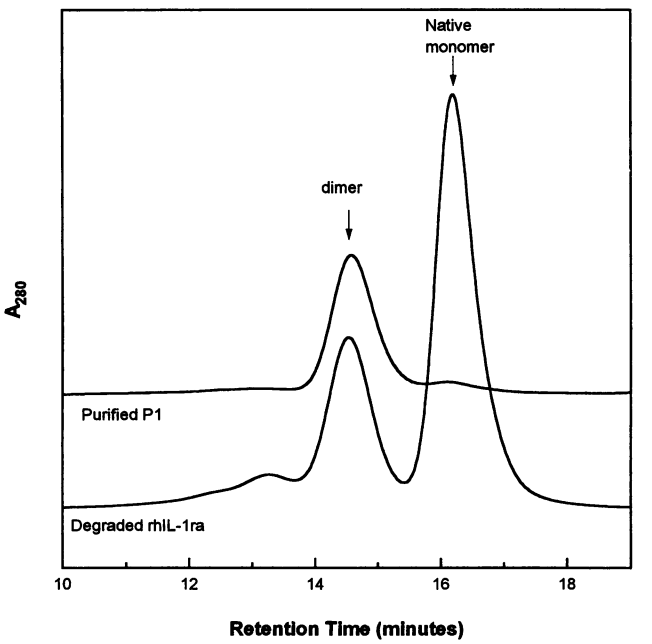

FIGURE 2 Analysis of purified P1 fraction by SEC-HPLC. The dimer shown in the chromatogram was confirmed using molecular weight standards.

As noted in the Introduction, irreversibly aggregated degradation products formed from proteins, which are structurally similar to rhIL-1ra, are largely unfolded, and often such aggregates contain a high degree of intermolecular β sheet structure (Dong et al., 1995). To determine whether this was true for the formation of a soluble dimer of rhIL-1ra, we examined the structure of the purified P1 fraction with a number of biophysical methods. First, the secondary structure of the native and dimeric protein was compared using infrared spectroscopy. The second derivative spectra in the conformationally sensitive amide I region (Susi and Byler, 1983) were very similar for the two protein forms (Fig. 3) and were characteristic of predominantly β sheet proteins (Dong et al., 1990; Dong and Caughey, 1994). The spectrum for the dimer had slightly greater absorbances for the bands at 1642 and 1691 cm^{-1}, which are due to intramolecular β sheet structure. This increase in absorbance was compensated by a slight reduction in absorbance in the β turn region of 1660–1685 cm^{-1} (Dong and Caughey, 1994). Also, the characteristic infrared bands for the intermolecular β sheet were not present in the spectra of either protein form (Fig. 3). For proteins prepared in H_2O, intermolecular β sheet gives rise to a strong band in the 1615–1625 cm^{-1} region and a weaker band in the 1690–1699 cm^{-1} region (Dong et al., 1995). Taken together, these infrared spectroscopic results demonstrate that the secondary structure of the P1 dimer is very native-like and has only a slight increase in intramolecular β sheet content relative to that of the native monomer.

These results were corroborated with CD spectroscopy. The far-UV CD spectrum (Fig. 4 *A*) of the P1 dimer was slightly different from that of the native protein, and indicated that the β sheet content of the secondary structure was only slightly increased in the P1 dimer relative to native, monomeric protein. Also, it appears that the environment of the aromatic amino acids responsible for the 231-nm peak were only slightly altered by dimer formation. The near-UV CD spectra (Fig. 4 *B*) did suggest the possibility of a small change in tertiary structure. The overall shape of the spectrum for the native protein, including features attributable to Trp (280–300 nm) and Tyr (270–280 nm), was retained in the spectrum for the P1 dimer, but the absolute intensity of the signal throughout the near-UV CD region was more positive in the P1 form. There also appeared to be a difference in the features at 250–260 nm, which could be attributable to an alteration in the local environment of Phe.

However, because disulfides have a broad signal in this spectral region, the changes in the spectrum could be observed if cysteines had formed a disulfide in the P1 dimer. Native rhIL-1ra contains no disulfide bonds. The four cysteines, Cys-66, Cys-69, Cys-116, and Cys-122 exist in a free form in the native protein (Eisenberg et al., 1990). The observation that the P1 dimer is dissociated during SDS-PAGE implies that disulfide bonds are not formed at the intermolecular contacts. However, tryptic mapping indicated that *intra*monomer disulfide bonds had formed in the

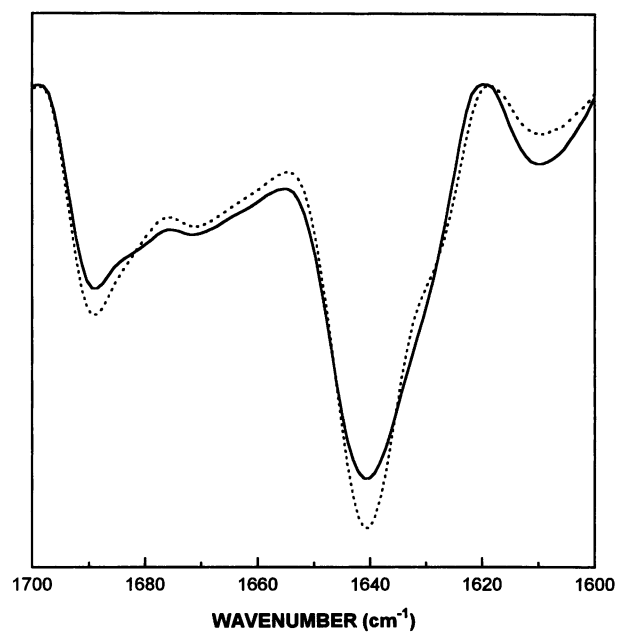

FIGURE 3 Second-derivative amide I infrared spectra of native rhIL-1ra (——) and the P1 dimer (·····).

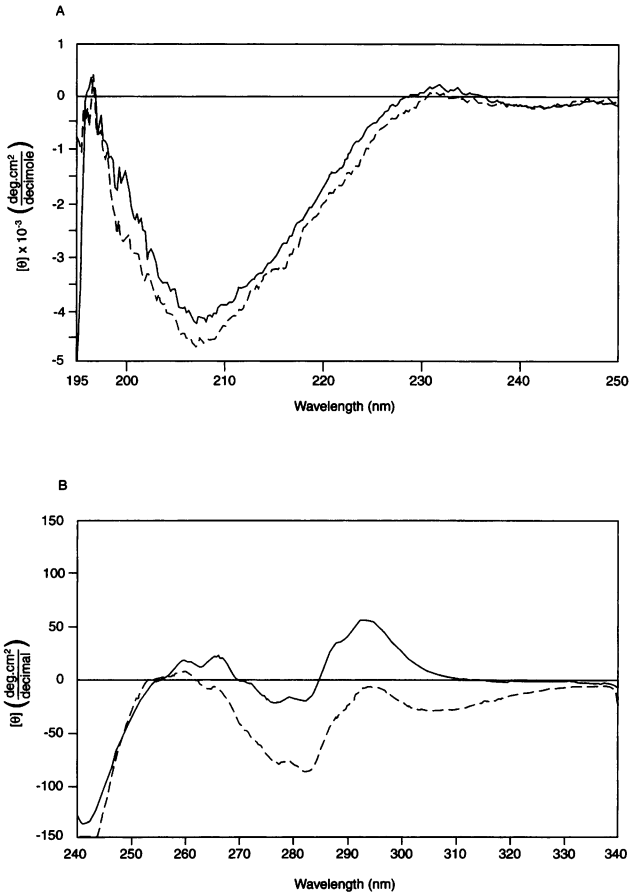

FIGURE 4 Circular dichroism analysis of native rhIL-1ra (——) and the P1 dimer (- - - -). (*A*) Far-UV analysis and (*B*) Near-UV analysis.

P1 dimer. Comparison of the tryptic map of the native protein and the P1 dimer showed a shift of one tryptic peak, T8, which contains Cys-66 and Cys-69 (data not shown). The two cysteines are present in free form in the native monomer and available for reaction during carboxymethylation. This treatment of the sample before trypsin digestion gave a molecular weight (by mass spectrometry) of the T8 peptide from native rhIL1-ra as 899 amu. In contrast, mass spectrometry analysis of the P1 dimer after the same treatment gave a molecular weight of 793 amu, which is less than the value for the T8 peptide from the native monomer by the mass of two carboxymethylated groups. This result indicates that Cys-66 and Cys-69 in the P1 degradation product were no longer in free form due to the presence of an intramonomer disulfide bond. Based on the proximity of these residues (Vigers et al., 1994), the formation of the disulfide bond is not unexpected and agrees with the observed change in the near-UV CD spectra upon dimer formation. However, as discussed below, this disulfide formation does not greatly reduce antagonistic potency activity of the P1 dimer, nor does it prohibit refolding of this species.

To probe further for conformational alterations in the P1 dimer, the overall surface hydrophobicity was assessed by ANS binding. ANS is a hydrophobic dye that fluoresces upon binding to hydrophobic patches on the surface of proteins (Semisotnov et al., 1991). Addition of up to 200 mM ANS to both the P1 dimer and the native monomer resulted in identical, very low, fluorescence at 500 nm (data not shown). This result indicates that both species have essentially equivalent, very hydrophilic exposed surfaces.

The structural analyses all indicated that the P1 dimer has a very native-like structure. To determine whether this structural retention corresponded to maintenance of biological activity of the protein, the in vitro antagonistic potencies of the P1 and L2 dimers were compared with those of the native monomer. The dose dependencies of competition with IL-1a are shown in Fig. 5. The shape of the fitted curves were very similar for all three samples, demonstrating that the P1 and L2 dimers competed with IL-1a in a similar fashion to the native monomer. The dose response curves for the dimers were shifted to about a threefold higher concentration, indicating that they are about one-third as active as the native protein. Because IL-1a is a monomeric ligand, only one subunit in the P1 dimer would be expected to bind the receptor. Hence the activity of P1 and L2 dimers is theoretically expected at most to be one-half that of the native monomer. The observed activity, therefore, indicates that on a per monomer basis the dimers retained about two-thirds of the activity of the native monomer.

All of these spectroscopic and biological data indicate that the P1 has a native-like folded structure that is only slightly different from that of the native monomer. There are two distinct possibilities as to how this novel degradation product is formed. First, the dimer could be formed from random contact between two native monomers in an appropriate orientation and, hence, the rate of formation of the P1 dimer should follow second-order kinetics. In this mechanism, the minor conformational changes (e.g., formation of intramonomer disulfide bond) would arise after dimerization. Second, there could be a transition of the native monomer into an intermediate, mostly folded state, which then associates into the dimer. In this case it is expected that rate of dimerization would follow first-order kinetics. To address this issue the time course of P1 dimer formation was compared with curves expected for first- and second-order reactions, as shown in Fig. 6. The results fit best with a first-order reaction, which is consistent with the hypothesis that a conformational change in the native monomer is responsible for P1 formation and is the rate-determining step.

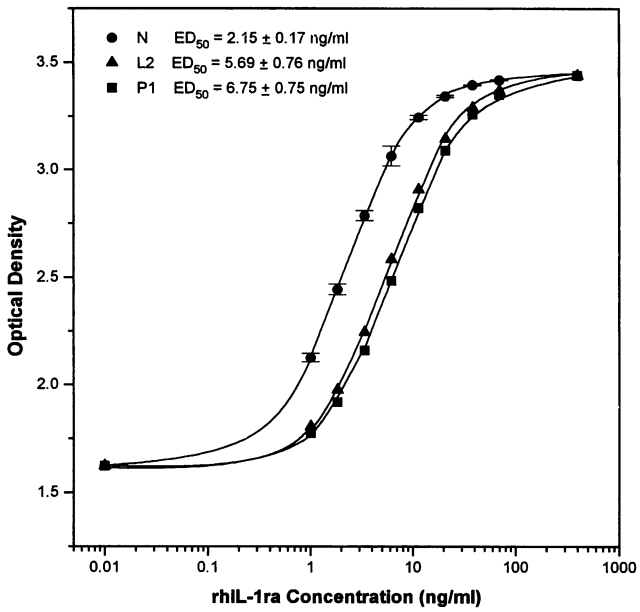

FIGURE 5 Potency bioassay of native rhIL-1ra and purified P1 or L2 dimers. ED_{50} values include their standard deviations.

Renaturation of the P1 dimer

The conversion of monomer to the P1 dimer is irreversible, as shown by the lack of appearance of the native monomer upon injection of purified P1 into a CEX-HPLC or a SEC-HPLC system (Figs. 1 and 2). However, the P1 dimer could be converted to the native monomer with a denaturation/refolding treatment. From the dose response for unfolding in the presence of guanidine hydrochloride, as assessed by alterations in intrinsic fluorescence, it was established that the midpoint for protein denaturation was 1.6 M guanidine hydrochloride and that the protein was fully denatured in 2.5 M guanidine hydrochloride (data not shown). Starting with the fully denatured form in 2.5 M guanidine hydrochloride, the protein was refolded by dialyzing it against 10 mM sodium citrate buffer with 140 mM NaCl at pH 6.5. The refolded material was analyzed by CEX-HPLC (Fig. 7 *A*).

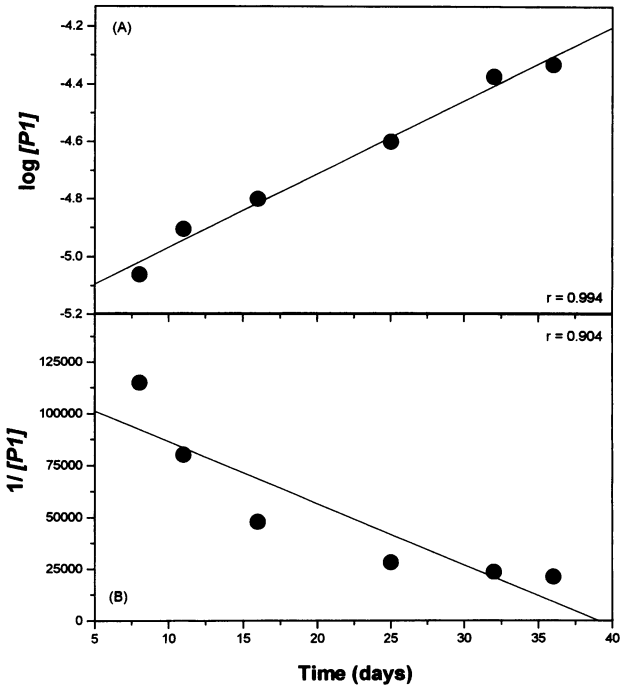

FIGURE 6 Fitting of data for P1 dimer formation as a function of storage time to equations for first-order (*A*) and second-order (*B*) reactions (see Methods).

The majority of the refolded protein eluted at the position for the native monomer, indicating that the intramonomer disulfide formation in the P1 form did not interfere with refolding. Surprisingly, a small peak was also observed at the P1 position, suggesting that the refolding process generates the P1 dimer as well as native monomer. When the native monomer was denatured with 2.5 M guanidine hydrochloride and refolded and analyzed as described above, it generated not only the native monomer but also a small amount of P1 dimer (Fig. 7 *B*). These results suggest that during the course of refolding from the denatured state the requisite monomeric conformation for formation of the P1 dimer can be generated.

Effect of sucrose on rhIL-1ra stability

Sucrose and other stabilizing co-solvents, which are known to increase the free energy of protein denaturation (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982, 1985; Timasheff, 1992, 1995; Arakawa et al., 1993), have been shown to reduce formation of non-native protein aggregates during storage in aqueous solution (Wilson, 1970; Frigon and Lee, 1972; Gu et al, 1991; Tsai et al, 1993; Chen et al., 1994). As noted in the Introduction, this effect is the result of making less favorable the denatured state needed for aggregate formation. In contrast, the formation of the P1 dimer does not appear to be associated with denaturation of the monomer. Because the dimer is mostly native in structure, it might be expected that the capacity of a co-solvent such as sucrose to foster polymerization of native proteins (cf. Timasheff 1992, 1995; Arakawa et al., 1993) could enhance the rate of production of the P1 dimer. However, the generation of P1 and L2 dimers during storage of rhIL-1ra was greatly reduced by the addition of sucrose in a concentration-dependent manner (Fig. 8). With sucrose con

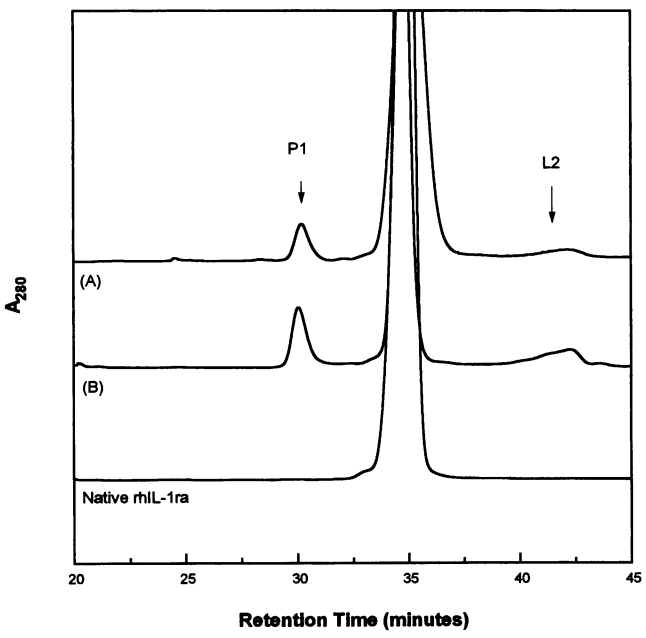

FIGURE 7 CEX-HPLC chromatograms of renatured P1 (*A*) and native rhIL-1ra (*B*). Each sample was denatured with 2.5 M guanidine hydrochloride and dialyzed against 10 mM sodium citrate buffer with 140 mM NaCl at pH 6.5.

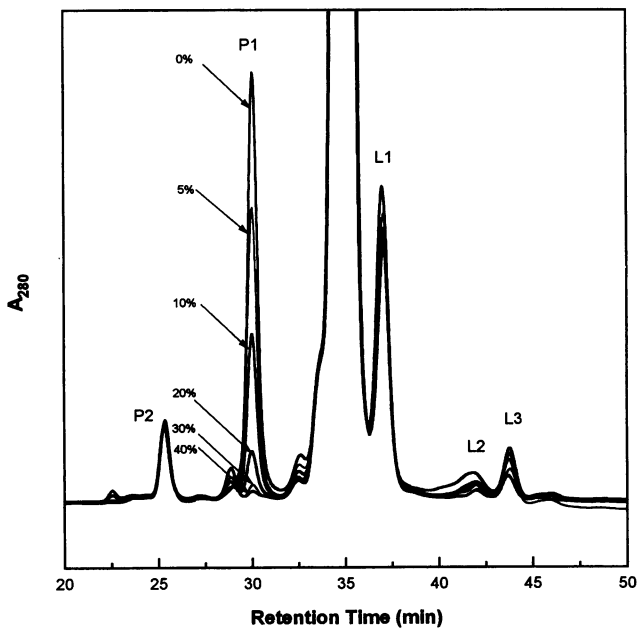

FIGURE 8 CEX-HPLC chromatograms of rhIL-1ra samples containing various sucrose concentrations after 2 months of storage at 30°C. Numbers shown in the figure represent the concentration of sucrose (w/v).

centrations ≥40% (w/v) these degradation products were almost undetectable after 2 months of storage at 30°C. The same trend was observed when sorbitol was included instead of sucrose (data not shown).

Possible mechanisms of P1 dimer formation and stabilizing effect of sucrose

Taken together, our results indicate that the overall structure of the dimeric P1 degradation product is only slightly altered relative to that of the native monomeric rhIL-1ra. The first-order reaction kinetics suggest that the rate-limiting step could be conversion of the monomeric native protein into a monomeric form with slightly altered structure, which serves as the precursor for formation of dimers. The observation that the structural stabilizer (sucrose) inhibits the formation of the P1 dimer also suggests that there must be at least some perturbation of conformation involved in the dimerization, based on the following arguments.

Timasheff and colleagues (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982) have shown that sucrose and other structural stabilizers exert their effects because they are preferentially excluded from the protein's surface, which increases protein chemical potential. By the principle of Le Chatelier, at equilibrium the system will tend to minimize this thermodynamically unfavorable effect by reducing the surface area of the protein from which the co-solvent is excluded. Thus, the protein state with the lowest surface area of exposure to the solvent will be favored. When a protein is in equilibrium between native and denatured states, in the presence of a preferentially excluded co-solvent, the increase in protein chemical potential is greatest for the latter state, which has the greatest surface area. Hence, the free energy of denaturation is increased and the native state is stabilized.

In the case of sucrose-induced inhibition of P1 dimer formation of rhIL-1ra, we propose that sucrose affects the reversible conversion of native monomer (*N*) to an intermediate form (*I*) in favor of *N*, because there is an increase in protein surface area upon formation of *I*. Also, Liu and Bolen (1995) have recently shown that the unfavorability of exposing the peptide backbone to solvent increases in the presence of sucrose. Such exposure could arise during formation of *I* in our system.

The inhibition of the formation of P1 dimer by sucrose also argues against dimerization from initially fully native molecules. This is because—also by the Principle of Le Chatelier—in the presence of a preferentially excluded co-solvent the formation of oligomeric species would be favored because the overall surface area of the protein molecules would be reduced by polymerization. Hence, even though the formation of the P1 dimer is an irreversible step, it would be expected that sucrose would accelerate this process if the native protein monomer was the form involved in dimerization.

To summarize, we suggest that the formation of the P1 degradation product occurs as follows:

$$N \rightleftarrows I \rightarrow \text{P1}$$

The native monomer is in equilibrium with a monomeric intermediate leading to P1, termed *I*. This conversion from *N* to *I* could itself be irreversible. However, the apparent stabilization of the native monomer by sucrose suggests that there may be an equilibrium step during P1 formation, as described in this scheme. To our knowledge there have not been other published reports documenting the formation of active, oligomeric degradation products of proteins by this or other pathways. More research with other proteins is needed to determine whether this behavior is unique to rhIL-1ra.

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