Disulfide Bridges in Interleukin-8 Probed Using Non-Natural Disulfide Analogues: Dissociation of Roles in Structure from Function†

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ABSTRACT: The structural and functional roles of the two disulfide bridges in interleukin-8 (IL-8) were addressed using IL-8 analogues with covalently modified disulfide bridges. The analogues were prepared using chemical synthesis by replacement of a cysteine for either homocysteine, penicillamine, or selenocysteine and on folding resulted in a covalently modified disulfide. Deletion of either of the two disulfide bridges by replacement of either cysteine pair with alanine resulted in loss of both structure and function. In contrast, all of the analogues with modified disulfide bridges had native tertiary fold as determined by nuclear magnetic resonance spectroscopic methods. Their structural similarity provided a rational basis for assessing the functional effects of the changes to the disulfide. Modification to the disulfide bridge between cysteines 9 and 50 had only a modest effect on IL-8 function. In contrast, alterations to the 7−34 disulfide bridge resulted in a dramatic reduction in biological potency. Thus, although both disulfide bridges are required for maintenance of the native tertiary fold, their role in determining IL-8 activity is distinct. We propose that 7−34 disulfide has a direct role in determining receptor binding and activation, whereas the 9−50 was not directly involved. The synthesis of non-natural disulfide analogues is a novel general approach to structure−activity relationships of disulfide bridges. The demonstration that the participation of disulfide bridges in function can be dissociated from their effects on the stability of the tertiary structure suggests that this method will lead to increased understanding of the roles of disulfide bridges in proteins.

Secreted globular proteins often contain disulfide bridges, which are essential for folding, stability, and tertiary structure, and the mechanism by which disulfide bridges contribute to folding and structure has been intensively studied (*1*−*4*). The importance of a disulfide bridge for biological functions is usually inferred by measuring the effect of deletion of the disulfide by replacement of the cysteine pair for alanines. This approach is limited because effects on function could be a consequence of the loss of tertiary structure, and thus determining the role of disulfides in protein function and structure represents a challenging problem for conventional mutagenesis approaches. We have approached the role of disulfide bridges in protein structure and function by synthesis of analogues with covalently modified cross-links which contain non-natural amino acids. In contrast to the deletion analogues, these analogues retain the original cross-link, but with a conservative change to its covalent structure. We hypothesize that this approach will allow a more quantitative evaluation of the role of disulfides than deletion approaches, and in particular help dissociate structural and functional roles.

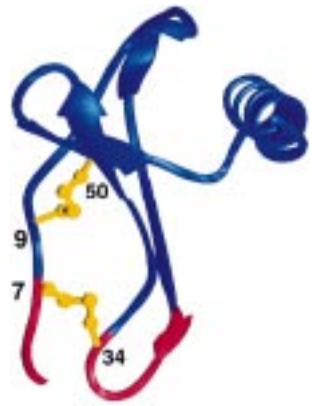

FIGURE 1: MOLSCRIPT (*23*) description of IL-8 structure and the topology of the disulfide bonds. The structure consists of three β strands (blue) and an overlying α helix (red). The two disulfide bonds, 7−34 and 9−50, are shown in yellow.

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[1] Abbreviations: EC-30, effective concentration for 30% of maximal response; IL-8, interleukin-8; HCys, homocysteine; Pen, Penicillamine; SeCys, selenocysteine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

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Table 1: Biological Potency and Receptor Binding of IL-8 Analogs

analog	relative activity[a]	relative binding[b] CXCR1	CXCR2
native [C_α-C_β-S-S-C_β -C_α]	1.0	1.0	1.0
HCys-7 [$C^7{}_\alpha$-C_β-C_γ-S-S-C_β -$C^{34}{}_\alpha$]	150 ± 71	83	83
HCys-34 [$C^7{}_\alpha$-C_β-S-S- C_γ-C_β -$C^{34}{}_\alpha$]	47 ± 21	20	42
HCys-9 [$C^9{}_\alpha$-C_β-C_γ-S-S-C_β -$C^{50}{}_\alpha$	2.9 ± 0.8	5	3
HCys-50 [$C^9{}_\alpha$-C_β-S-S- C_γ-C_β -$C^{50}{}_\alpha$]	3.6 ± 0.2	4.2	5
Pen-7 [$C^7{}_\alpha$-$C_\beta(CH_3)_2$-S-S-C_β -$C^{34}{}_\alpha$]	415 ± 301	nd	
Pen-34 [$C^7{}_\alpha$-C_β-S-S-$C_\beta(CH_3)_2$-$C^{34}{}_\alpha$]	65 ± 31	nd	
SeCys-7 [$C^7{}_\alpha$-C_β-Se-S-C_β -$C^{34}{}_\alpha$]	2.0 ± 0.4	nd	
SeCys-34 [$C^7{}_\alpha$-C_β-S-Se-C_β -$C^{34}{}_\alpha$]	1.7	nd	
SeCys-9 [$C^9{}_\alpha$-C_β-Se-S-C_β -$C^{50}{}_\alpha$]	1.1	nd	

[a] Relative elastase release activity is the EC_{30} of the analogue, divided by the EC_{30} of native IL-8. The EC-30s were determined from tirations of each sample; representative elastase release data is shown in Figure 5. Results are expressed as the mean ±sd of 4−10 experiments. Those with out an sd value were done twice. [b] The dissociation constant (K_d) was measured by competition of the analogue for the binding of ^{125}I-radiolabeled IL-8 to either CXCR1 or CXCR2 receptors expressed in HEK293 cells. For direct comparison with the elastase release results, the analogues were normalized to the K_d of native IL-8. The values are the means of two similar experiments.

Human interleukin-8 (IL-8)[1] is a member of the chemokine family of pro-inflammatory proteins and promotes the chemotaxis and activation of neutrophil leukocytes. It is a 72 amino acid residue protein and has two disulfide bridges, from Cys-7 to Cys-34 and from Cys-9 to Cys-50 (*5*, *6*). Both disulfide bridges are critical for function, because disruption of either one resulted in loss of activity (*7*). Two regions, which are linked by the Cys-7-Cys-34 disulfide bond and form a contiguous surface, are critical for function. These regions are N-terminal residues Glu-4, Leu-5, and Arg-6, and a β-turn containing Gly-31 and Pro-32 (Figure 1) (*7*−*9*). Previously, we proposed that the core of the IL-8 protein acts as a scaffold for the functionally important, but mobile, N-terminal residues so that they are oriented for optimal interaction with the receptor (*8*). The two disulfide bridges link the N-terminal region to the core of the protein and therefore form a critical part of the protein scaffold.

In this study we begin to address the roles of the disulfide bridges in function. For example, they could be directly involved in receptor binding, or they could link domains that are critical for receptor interaction. We approached this problem by chemically synthesizing a series of IL-8 variants with one of the four cysteines substituted with a cysteine analog: either homocysteine (HCys), penicillamine (Pen), or selenocysteine (SeCys) (Table 1). These analogues had one covalently modified, asymmetric disulfide cross-link and one native disulfide bridge. Moreover the solution structure of the analogues was indistinguishable from that of the native molecule. Thus we have overcome the major barrier to analysis of disulfides: generating modified forms with the normal tertiary structure. On the basis of the structural findings, the effects on the biological activities were compared. The implications for the local effects on function are discussed.

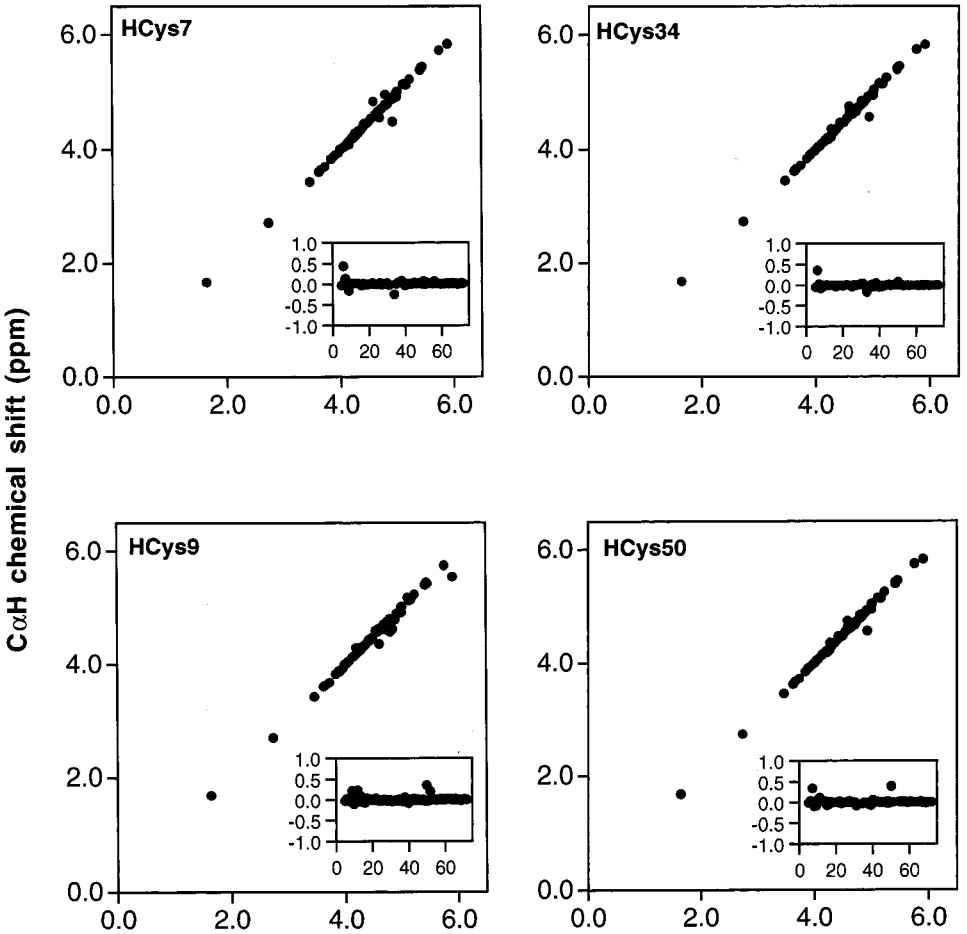

FIGURE 2: Plots of the ^{1}H NMR chemical shift of the C_αHs of the native and the HCys analogues. The chemical shifts of the native protein are plotted along the *y*-axis. The differences in the ^{1}H NMR chemical shift of the C_αH between the native and the different HCys analogues versus the residue number is shown in the inset.

MATERIALS AND METHODS

Chemical Synthesis of IL-8 Analogues. We had shown previously that a shortened form of IL-8 (IL-8, 4−72), and which had the ELR motif at the N-terminus, was more potent than the 1−72 form. Therefore the analogues in this study, as with previous structure−function studies (*7*−*9*), correspond to the 4−72 form. Solid-phase peptide synthesis was carried out using the tBoc protection strategy on an 430A peptide synthesizer (Perkin-Elmer-Applied Biosystems, Foster City, CA) (*10*). The synthesis was started with the C-terminal serine linked to a pam resin support, and amino acids were coupled using either active esters, formed with HBTU, or symmetrical anhydrides (*10*, *11*). Similar results were obtained with either chemistry. In both cases a protocol was used where activated amino acid was added to the peptide resin twice to improve the yields. L-HCys and L-Pen (β,β-dimethylcysteine) were incorporated as the N^{α} tBoc and *S*-(4-methylbenzyl) protected form (Bachem Biosciences, King of Prussia, PA). Protected L-SeCys was prepared from β-L-chloroalanine (Aldrich Chemical Co., Milwaukee, MI) by reaction with sodium diselenide (Aldrich), and the Se was protected first using 4-methylbenzylbromide and the $^{\alpha}$N with tBoc anhydride, as described (*12*). The analogues were deprotected using the low−high hydrogen fluoride method as described (*11*), then folded using 10% v/v in water, and purified by reverse-phase HPLC on preparative (22 × 250 mm) and semipreparative (10 × 250 mm) C18-silica columns (Vydac, Hesperia, Ca) (*10*). Each analogue was analyzed by electrospray mass spectrometry on a API 300 triple quadrapole mass spectrometer (Perkin-Elmer-SCIEX, Thornhill, ON) to verify that the molecular weight was consistent with the target protein. For each analogue the measured mass ±sd, followed by the calculated mass, was the following: HCys-7, 8109.35 ± 0.82, 8109.51; HCys-34, 8109.45 ± 0.77, 8109.51; HCys-50, 8109.35 ± 0.82, 8109.51; HCys-9, 8109.45 ± 0.91, 8109.51; Pen-7, 8123.21 ± 1.04, 8123.54; Pen-34, 8124.31 ± 1.17, 8123.54; SeCys-7, 8142.38 ± 1.4, 8142.37; Se Cys-34, 8142.62 ± 0.58, 8142.37. The results were consistent with the formation of the disulfides (or selenosulfide) because each had around 4 amu lower mass than that expected for the four SH reduced form. Each purified protein was analyzed by reverse-phase HPLC and by cation-exchange HPLC to help us verify their purity. For each protein all of the residues were explicitly assigned by NMR, and the presence of the S−S or S−Se bond was unequivocal.

NMR Spectroscopy. ^{1}H NMR spectra were acquired on a Varian Unity 600 spectrometer at 30 and 40 °C as described previously (*8*). The protein concentration was 2 mM in 20 mM sodium acetate, pH 5.2, in 90% H_2O/10% $^{2}H_2O$(v/v). The chemical shifts and the NOE assignments were made by use of NOE and exchange spectroscopy (*13*) (mixing time 150 ms) and total correlated spectroscopy (*14*) (mixing time 55 ms) experiments. Water suppression was accomplished using gradients and the WATERGATE scheme (*15*).

IL-8 Assays. Human neutrophils were purified from donor blood, and assays for IL-8-stimulated elastase release from neutrophils were performed as described (*16*). Competition binding studies were carried out using iodine-125-labeled IL-8 and transfected HEK 293 cells expressing either CXC chemokine receptor 1 or CXC chemokine receptor 2 (*17*).

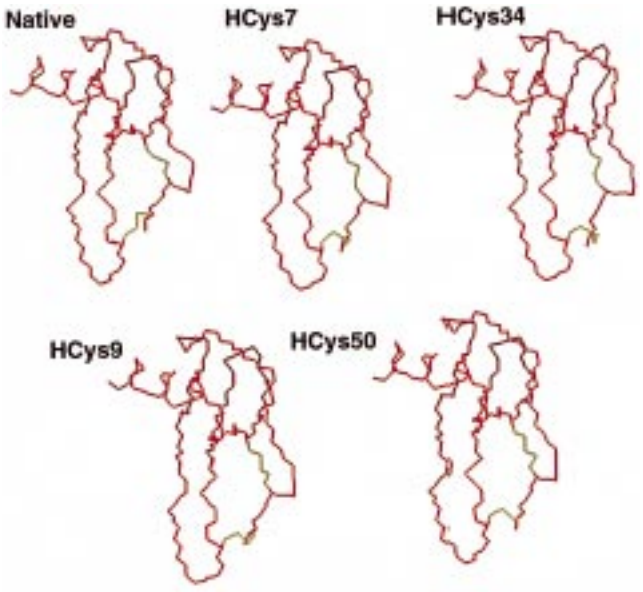

FIGURE 3: The tertiary fold of the four HCys analogues is similar to that of the native protein. Shown is the backbone trace of the minimized NMR structure of the HCys analogues. The coordinates of the monomeric IL-8 structure (*19*) were used to study the effect of replacing each of the four cysteines with HCys. As the NOE pattern was essentially the same in all of the analogues, the same distance restraints were used in all of the structure calculations. Thirty structures were generated for each of the homocysteine analogues using the program X-PLOR (*24*). All structures showed good covalent geometry, and there were no distance violations greater than 0.3 Å. The root-mean-square-deviation (rmsd) of the 30 structures to the average structure was less than 0.6 Å for the backbone atoms for residues 7−66.

RESULTS

NMR Analysis of the Disulfide Analogues. The structures of native IL-8 (*18*), an IL-8 monomer (*19*), and various analogues (*8*) have previously been determined by NMR spectroscopy. The structure calculations were based on measurement of the nuclear Overhauser effect (NOE), which provided relative distances of protons in the context of the tertiary structure. For example, NOEs were observed in the between protons of Leu-25 and Val-41; Val-27 and Ile-39; Ile-40 and Cys-50; Lys-42 and Glu-48; and Val-41 and Leu-49, due to the involvement of these residues in β sheet formation. NOEs were also observed across the dimer interface, indicating that the molecule is present in its dimer form (*18*). NOEs were identified between Thr-12 and Cys-50; Tyr-13 and Leu-49; Phe-17 and Val-61; Trp-57 and Tyr-13, Ser-14, Pro-16, and Phe-17; and Phe-65 and Lys-23, Glu-24, and Leu-25, due to the proximity of these residues in the folded tertiary structure. Essentially the same set of NOEs were observed in all four HCys-containing IL-8 analogues, indicating that the modified disulfide bridge is accommodated into the protein structure without affecting the fold of the native molecule. There is similarity in the NOE pattern of the HCys-7 analogue and the native protein (see Supporting Information). In the native protein and HCys analogues, residues in the vicinity of the 7−34 disulfide show only a few intraresidue and sequential NOEs, suggesting that this region lacks well-defined structure.

Chemical shifts are sensitive to secondary and tertiary structure, and those which are characteristic of the IL-8 tertiary structure belong to the Val-58 spin system (NH, 5.79

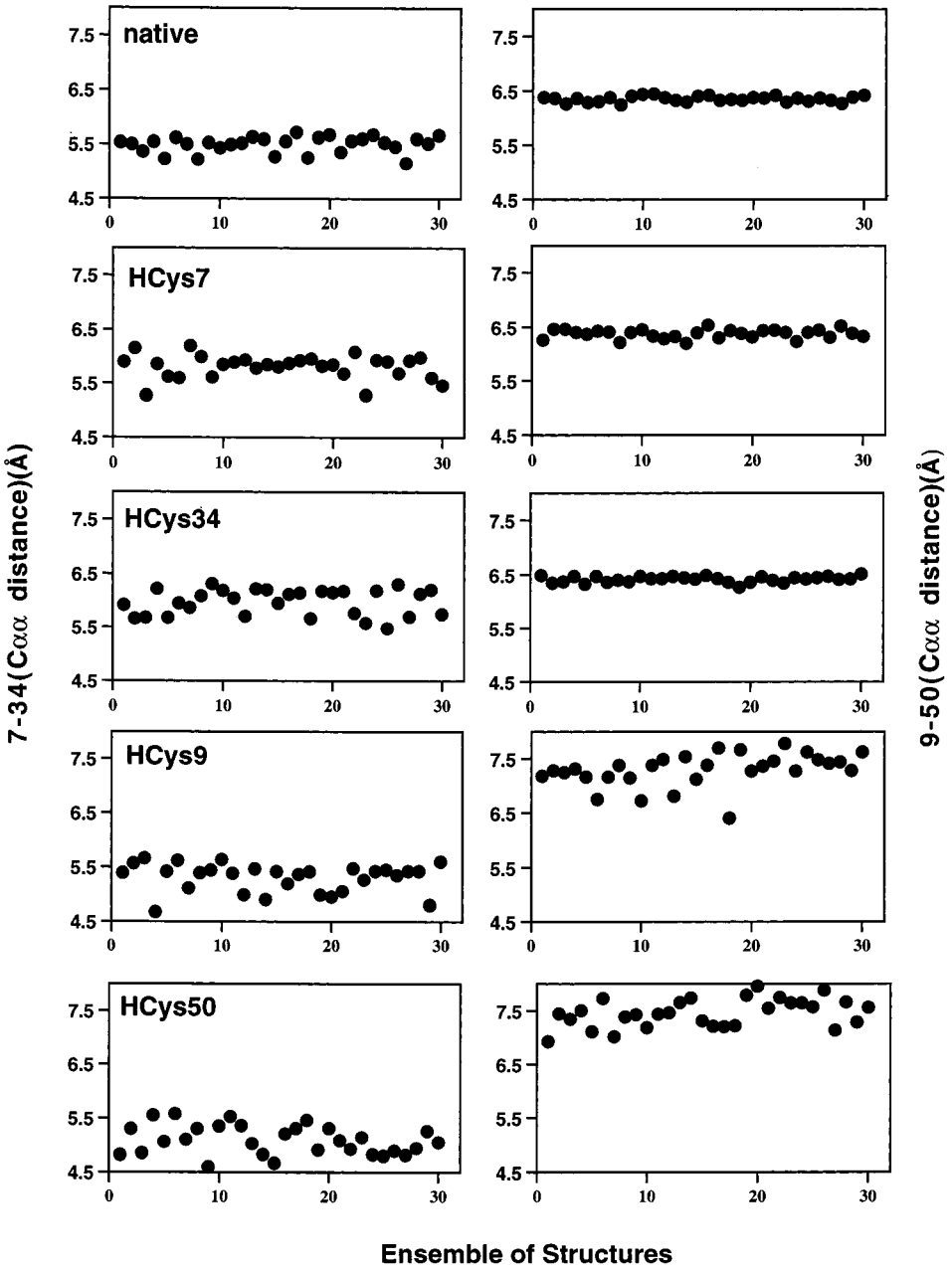

FIGURE 4: The effect of HCys substitutions on the $C_{\alpha\alpha}$ distances of the disulfide bridges. The distances for the two disulfide bridges (7−34, left panels, and 9−50, right panels) were calculated from the structures of all of the four HCys analogues.

ppm; CαH, 2.72 ppm; CβH, 1.94 ppm; CγH_3, 0.35 ppm, −0.42 ppm). Some shifts are the following: Lys-15 NH, 6.32 ppm; Pro-16 CαH, 1.64 ppm; Phe-17 NH, 5.90 ppm; and Cys-50 CαH, 5.91 ppm. Similar chemical shifts were observed for these protons in the cysteine analogues. Previous studies have shown that the CαH chemical shift is sensitive to differences in the tertiary structures of single-substitution analogues. The chemical shifts were identical through most of the molecule, and the very small differences were confined to the site of the substitution (Figure 2). This indicates that the structures of the analogues are similar to the native protein. Chemical shift comparisons are especially useful for less-structured regions of the protein where there is a paucity of NOEs. For example, the backbone amide proton of Gln-8 (the residue which is located between Cys-7 and Cys-9) has a pronounced downfield shift due to hydrogen bonding with the carboxylate of Glu-38 (*20*). In all four HCys analogues, the Gln-8 NH shifts were still significantly downfield shifted, suggesting that the hydrogen bond is retained. Overall, the data indicate that the small, local structural changes are attributed directly to the covalent modification itself, rather than changes in the global tertiary fold.

The disulfide bridges in the HCys-7 and-HCys-34 analogues have an extra methylene group compared to the native disulfide and thus would be expected to form a longer cross-link. However, in the tertiary structure, the length of the disulfide bridge is also dependent on its conformation, which is determined by other interactions. Therefore we analyzed the distance between the two Cαs of the modified disulfide bridges in the analogues. The four HCys disulfide analogues were modeled on the structure of IL-8 monomer, and an ensemble of 30 structures of each analogue was generated (Figure 3). This allowed a statistical analysis of the range of the disulfide lengths that were adopted in the structures

(Figure 4). The two 9−50 disulfide bridge analogues (HCys-9 and HCys 50) were, on the average, 1 Å longer than the native 9−50 disulfide bridge. However, the two 7−34 disulfide bridge analogues (HCys-7 and HCys-34) were only 0.4 Å longer than the native 7−34 disulfide.

To further test the role of the disulfides in IL-8 structure−activity relationships, we prepared analogues with alternative non-natural cysteine replacements. These analogues had either Pen-7, Pen-34, SeCys-7, or SeCys-34. The Pen analogues have bond lengths similar to that of the normal disulfide but are sterically hindered due to the two methyl groups on the β carbon. SeCys was the most conservative replacement, and the resulting seleno-sulfide cross-link would be expected to be similar in length and have degrees of freedom similar to those of the normal disulfide. NMR spectroscopic analysis indicated that, like the HCys analogues, the Pen and SeCys analogues also had a tertiary structure similar to that of native IL-8.

Functional Activities of the Disulfide Analogues. The biological activity of the analogues was compared to the native protein by assaying the IL-8-dependent release of elastase activity from human neutrophils (Figure 5, Table 1). The effective concentration required for 30% of a maximal response (EC30) and relative potency of the analogues is shown in Table 1. Also shown is the potency of the analogues with HCys at position 9 or 50 by a modest extent (~3-fold). In contrast, the HCys-7 and HCys-34 analogues had 150- and 45-fold lower potency, respectively. Previously characterized analogues that lacked a disulfide bridge lacked detectable activity (*7*). Receptor binding assays using cell lines expressing either CXCR1 or CXCR2, the two receptors for IL-8, paralleled the functional activities (Table 1). The Pen analogues of disulfide 7−34, like the corresponding HCys analogues, had a dramatically reduced potency and EC-30 (Figure 5, Table 1). These results, and those with the HCys analogues, indicate that changes to the flexibility and/or length of the cross-link affect the functional activity. In contrast, the SeCys analogues had activity comparable to that of native IL-8. This change to a selenosulfide bond instead of the normal disulfide bond is the most conservative modification that we evaluated. Nevertheless the results indicate that the 7−34 disulfide bridge can be modified without loss of function.

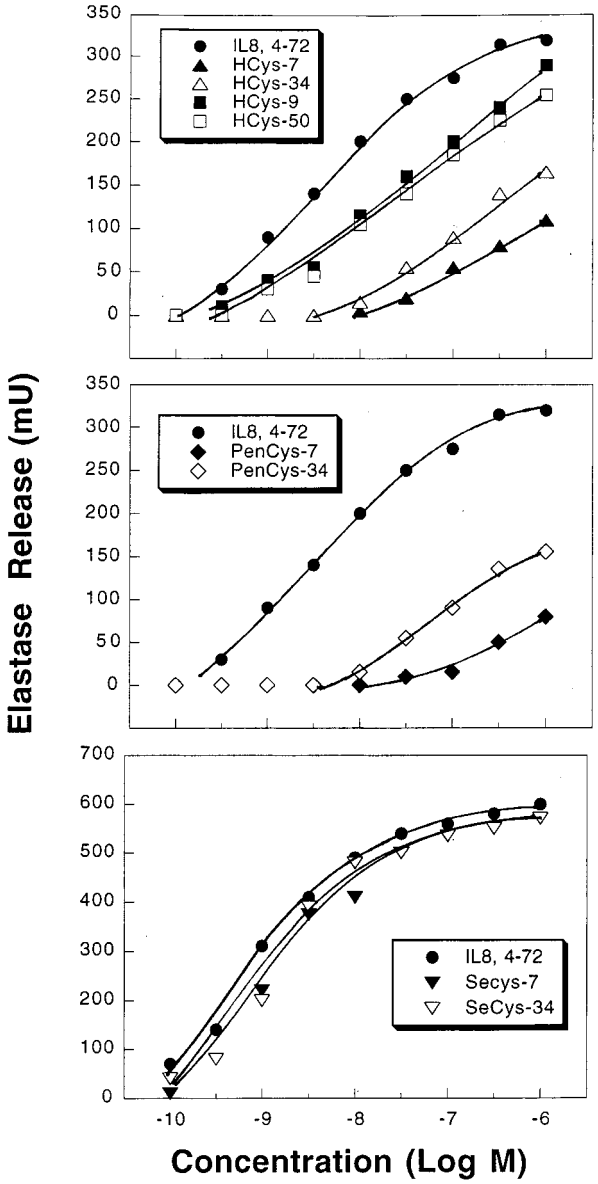

FIGURE 5: Elastase release activity. The indicated concentrations of each protein were added to cytochalasin B-pretreated human neutrophils, and after 6 min the elastase activity that was released into the medium was assayed. The data in the top and middle panels are from one experiment, and the data in the bottom panel is from a separate experiment.

DISCUSSION

In this study we have shown that the role of the two disulfide bridges is distinct. Whereas both disulfide bridges are essential for the tertiary fold, only the 7−34 disulfide is critical for function. NMR analysis demonstrated that the tertiary structures of the analogues with modified disulfide bridges were similar to that of the native protein. The modeling studies indicated that for the analogues with HCys-Cys disulfide bridges, the average length of the cross-links differed. In HCys-9 and HCys-50, the 9−50 cross-link was extended relative to the native form. This could be attributed to packing constraints on residue 50. In contrast, the 7−34 cross-link, as well as adjacent residues, is not constrained by close packing and is mobile in both native IL-8 and the analogues. Analysis of disulfide bridges in a set of high-resolution crystal structures has shown that Cα distances can vary from 3.8 to 6.8 Å (*21*), and this wide range in lengths reflects the geometric flexibility of the five different torsional angles within a disulfide bridge. Williams observed that disulfide bonds often link elements of structure which are highly mobile, and give an elastic quality to the protein, which could allow it to deform reversibly under stress (*22*). Our observation that the HCys analogues retain the tertiary structure of the native protein is consistent with this notion of elasticity. In particular, the lack of restraints for the 7−34 disulfide bridge allow it to accommodate the extra methylene group. Disulfide modification had no effect on the stability of the analogues as measured by thermal denaturation monitored by circular dichroism spectroscopy. Native IL-8 is highly stable and is structured up to 80 °C, and all of the HCys analogues showed denaturation profiles and stability properties similar to those of the native protein (data not shown).

For the HCys analogues of the 9−50 disulfide, despite the 1 Å increase in length of the cross-link, both the tertiary structure and functional activity were similar to the native protein. Since residue 50 is restrained and is part of the protein core, it is likely that the largest changes occur in residue 9 and suggests that the tertiary structure compensates for the greater length of the HCys-modified disulfide bridge. In contrast, the potency of the 7−34 disulfide analogues with HCys or Pen was severely compromised. The overall tertiary structure of the different analogues was maintained, and the region of the structure around the 7−34 is dynamic and is similar to the native protein.

The 7−34 disulfide is adjacent to the ELR motif (residues 4−6), which is critical for receptor binding and function (*9*). If the role of the 7−34 disulfide is to cross-link the ELR to the 31−34 turn and therefore purely structural, then substitution of either cysteine 7 or 34 would have been expected to result in a similar loss of activity. The observation that the HCys-7 and Pen-7 analogues were lower in potency than the HCys-34 and Pen-34 analogues suggests that the modified disulfides affect the binding of the ELR motif. Arg-6, which is adjacent to Cys-7, is the most critical ELR residue for function (*9*), and it is possible that modification to Cys-7 affects Arg-6 binding by steric effects. Alternatively, because the binding domain probably adopts a stable conformation on binding to the receptor, the 7−34 disulfide itself could make specific binding contacts with the receptor, thus accounting for the reduced effectiveness of the analogues. It is likely that receptor activation is a culmination of a number of specific and sequential steps. For example, after initial binding, the extra methylene group of the HCys, or the methyl groups of the Pen, could become exposed and result in steric hindrance. The more conservative change of a disulfide to a seleno-sulfide could affect a direct interaction of the disulfide with the receptor, but not the indirect interactions of the ELR motif.

We have shown that IL-8 analogues with non-natural disulfide bridges produced by chemical synthesis did not affect the tertiary structure of the protein but allowed the function of the disulfides to be evaluated. As the cysteines are conserved in the chemokines, as is the structural fold, the findings could be applicable to other chemokines (*5*). Replacement of the disulfide bridges in proteins with non-natural disulfide variants could be a general approach to the structure−activity relationships of disulfides in proteins. In the case of IL-8, structural requirements for function could be inferred from these analogues in a way similar to the way that conventional amino acid replacement mutants are compared. Further studies using this approach could contribute to our understanding of the complex interplay between protein structure, dynamics, folding, stability, and function.

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SUPPORTING INFORMATION AVAILABLE

Two figures of NMR data showing comparison of NOEs between native IL-8 and HCys IL-8 analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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