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Intrachain disulfide bond in the core hinge region of human IgG4

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Abstract

IgG is a tetrameric protein composed of two copies each of the light and heavy chains. The four-chain structure is maintained by strong noncovalent interactions between the amino-terminal half of pairs of heavy–light chains and between the carboxyl-terminal regions of the two heavy chains. In addition, interchain disulfide bonds link each heavy–light chain and also link the paired heavy chains. An engineered human IgG4 specific for human tumor necrosis factor-α (CDP571) is similar to human myeloma IgG4 in that it is secreted as both disulfide bonded tetramers (approximately 75% of the total amount of IgG) and as tetramers composed of nondisulfide bonded half-IgG4 (heavy chain disulfide bonded to light chain) molecules. However, when CDP571 was genetically engineered with a proline at residue 229 of the core hinge region rather than serine, CDP571(S229P), or with an IgG1 rather than IgG4 hinge region, CDP571(γ1), only trace amounts of nondisulfide bonded half-IgG tetramers were observed. Trypsin digest reverse-phase HPLC peptide mapping studies of CDP571 and CDP571(γ1) with on-line electrospray ionization mass spectroscopy supplemented with Edman sequencing identified the chemical factor preventing inter-heavy chain disulfide bond formation between half-IgG molecules: the two cysteines in the IgG4 and IgG1 core hinge region (C̲PSC̲P and C̲PPC̲P, respectively) are capable of forming an intrachain disulfide bond. Conformational modeling studies on cyclic disulfide bonded CPSCP and CPPCP peptides yielded energy ranges for the low-energy conformations of 31–33 kcal/mol and 40–42 kcal/mol, respectively. In addition, higher torsion and angle bending energies were observed for the CPPCP peptide due to backbone constraints caused by the extra proline. These modeling results suggest a reason why a larger fraction of intrachain bonds are observed in IgG4 rather than IgG1 molecules: the serine in the core hinge region of IgG4 allows more hinge region flexibility than the proline of IgG1 and thus may permit formation of a stable intrachain disulfide bond more readily.

Keywords: disulfide bonds; hinge region; IgG4; on-line electrospray ionization mass spectroscopy; peptide mapping

IgG is a tetrameric protein composed of two copies each of the light and heavy chains. Interchain disulfide bonds link each heavy–light chain and also link the paired heavy chains (Dorrington & Tanford, 1970; Dorrington, 1978; Burton, 1985). The inter-heavy chain disulfide linkage of monoclonal human IgG4 has been shown to be more sensitive to reducing agents than the heavy–light chain bridge. In contrast, the inter-heavy and heavy–light chain disulfide bridges of the IgG1, IgG2, and IgG3 subclasses were found to be equally sensitive to reduction (Virella & Parkhouse, 1973). The component heavy and light chains of human myeloma IgG4 have been found to assemble more slowly and with different intermediates than those of IgG1 in an in vitro system. The predominant IgG4 intermediate was heavy chain disulfide bonded to light chain (half-IgG), and two IgG1 intermediates were observed: half-IgG and half-IgG disulfide bonded to heavy chain (Petersen & Dorrington, 1974).

The four-chain IgG structure is also maintained by strong noncovalent interactions between the amino-terminal half of pairs of heavy–light chains and between the carboxyl-terminal regions of the two heavy chains (Dorrington & Tanford, 1970; Dorrington, 1978; Burton, 1985). Experiments with recombinant and recombinant/chimeric constructs of monoclonal antibodies have shown that antibodies bearing hinge regions of the γ4 subclass (IgG4) are secreted as both disulfide bond-linked tetramers and as half-IgG

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molecules assembled into tetramers by noncovalent interactions (Morrison et al., 1988; Colcher et al., 1989; Tan et al., 1990; Canfield & Morrison, 1991; King et al., 1992; Angal et al., 1993). The percentage of tetramers consisting of noncovalently associated half IgG molecules varies from 5 to 10% of the total secreted IgG (Colcher et al., 1989; King et al., 1992). Noncovalently associated tetramer dissociates into half-IgG molecules on nonreducing SDS-PAGE gels and separates from the antibody in which the inter-heavy chain disulfide bridges were formed. However, the noncovalent interactions between two half-IgG molecules are not overcome by gel-filtration HPLC or by native PAGE (Colcher et al., 1989; Canfield & Morrison, 1991; King et al., 1992). High-resolution ion-exchange chromatography can effect partial separation of the noncovalently associated half IgG molecules from the tetramer with inter-heavy chain disulfide bonds (Angal et al., 1993).

The amino acid sequence found within the core hinge regions of the IgG subclasses differ at position 241: CPX241CP (Kabat numbering system; Kabat et al., 1987). Angal et al. (1993) reasoned that the presence of a serine at position 241 of IgG4 rather than the proline seen in IgG1 and IgG2 might be related to the presence of noncovalently associated half-molecules in preparations of IgG4. Substitution of Pro 241 for Ser 241 led to the secretion of a homogeneous mouse/human chimeric IgG4 [cB72.3(γ4)] with inter-heavy chain disulfide bonds.

In the present report, we describe studies directed toward identification of the chemical factors that prevent inter-heavy chain disulfide bond formation in a significant fraction of IgG4 molecules with Ser 241. An engineered human IgG4 antibody to human tumor necrosis factor-α (CDP571; Dhainaut et al., 1995; Rankin et al., 1995; Stephens et al., 1995) was compared with two genetically engineered forms of the antibody: CDP571(S229P), CDP571 with a proline at residue 229 (equivalent to residue 241 in the Kabat numbering system; Kabat et al., 1987) of the core hinge region rather than serine, and CDP571(γ1), CDP571 with an IgG1 (EAPKSCDKTHTCPPCP) rather than IgG4 (ESKYGPPCPSCP) hinge region. Our results indicate that the two cysteines in both the IgG4 and IgG1 core hinge regions (CPSCP and CPPCP, respectively) are capable of forming both an intrachain disulfide bond and an interchain bond connecting heavy chains. However, a significantly greater proportion of intrachain disulfide bonds were found in molecules containing the IgG4 core hinge region relative to the levels in the mutant species containing the IgG1 core hinge.

Results

Antibody sequence confirmation

In order to confirm the sequences of CDP571(S229P) and CDP571(γ1), trypsin digest peptide maps of these two antibodies were compared with a map of CDP571 (not shown). As predicted, the maps were nearly identical and analysis of the differences in these maps by on-line electrospray ionization mass spectroscopy (ESI-MS) verified that the sequences of the proteins were correct (data not shown).

SDS-PAGE

The susceptibilities of interchain disulfide bonds in CDP571, CDP571(S229P), and CDP571(γ1) to reduction were determined by SDS-PAGE of the antibodies in the presence of varying amounts of DTT (Fig. 1). The presence of an intense half-IgG band that migrates with the 97-kDa standard at low DTT concentrations in the gel of CDP571 is the primary difference between the SDS-PAGE band patterns of the three antibodies. Densitometry analysis of these gels indicates that approximately 25% of the CDP571 preparation in 0.015 mM DTT is half-IgG, whereas CDP571(S229P) and CDP571(γ1) contain only trace amounts of half-IgG. The relative intensity of each half-IgG band is plotted versus DTT concentration in Figure 2. The intensity of the CDP571 half-IgG band reaches a maximum at a significantly lower DTT concentration than the other two antibodies. These results indicate that, without a reducing agent such as DTT, a significant fraction of CDP571 contains noncovalently associated half-IgG molecules and that the inter-heavy chain disulfide bonds of CDP571 are more sensitive to reduction than those of CDP571(S229P) and CDP571(γ1).

Peptide mapping

Trypsin digest reverse-phase HPLC (RP-HPLC) peptide mapping studies of CDP571 and CDP571(γ1) with on-line ESI-MS supplemented with Edman sequencing were used to identify the chemical factor preventing inter-heavy chain disulfide bond formation between half-IgG molecules. Two different methods of sample preparation before trypsin digestion were used in these experiments. In the first, the proteins were denatured by heating at 100 °C in 6 M guanidine in the presence or absence of DTT, followed by labeling of free cysteines with 4-vinylpyridine. In the second, the native

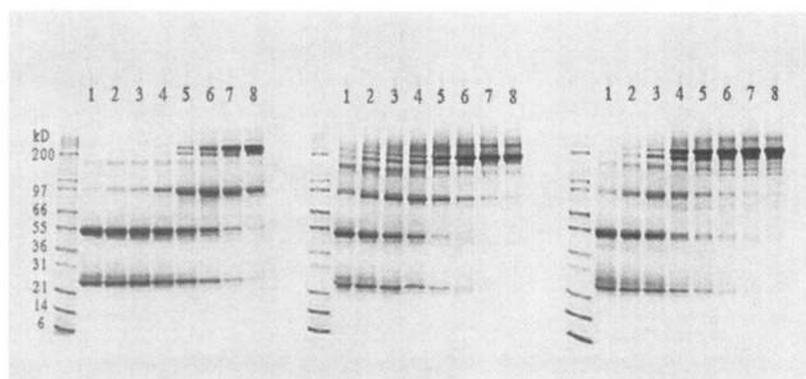

Fig. 1. Reduction of disulfide bonds by DTT. SDS-PAGE analysis of: CDP571 (left), CDP571(S229P) (middle), and CDP571(γ1) (right). Lanes 1–8 in each panel represent antibody treated with different concentrations of DTT: 1, 5 mM; 2, 1 mM; 3, 0.5 mM; 4, 0.25 mM; 5, 0.125 mM; 6, 0.062 mM; 7, 0.031 mM; and 8, 0.015 mM. A molecular weight standard was run in the left lane of each gel. The unreduced IgG, half IgG, heavy chain, and light chain bands migrate near the 200-kDa, 97-kDa, 55-kDa, and between the 31-kDa and 21-kDa standards, respectively.

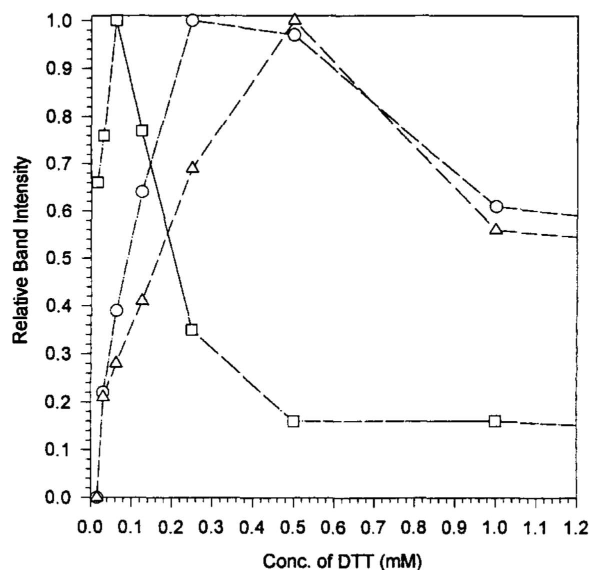

Fig. 2. Densitometric analysis of the data in Figure 1. Relative intensities of the half-IgG band, which migrates near the 97-kDa standard, are plotted versus DTT concentration for CDP571 (□), CDP571(S229P) (○), and CDP571(γ1) (△).

proteins were incubated in the presence or absence of DTT, followed by 4-vinylpyridine labeling of free cysteines before guanidine/heat denaturation.

Reduction and alkylation of denatured CDP571 and CDP571(γ1)

Denatured CDP571 and CDP571(γ1) samples with and without 50 mM DTT (with 4-vinylpyridine labeling of free cysteines) were compared by trypsin digest RP-HPLC peptide mapping. In the 0-mM DTT map of CDP571 (Fig. 3), a large peak at 118 min was found to contain a peptide with a measured mass of 2,827.5. This mass matched that of the heavy chain hinge region peptide YGPPC̲PSC̲PAPEFLGGPSVFLFPPKPK with an intrachain disulfide bond (residues 223–249, calculated masses of 2,829.4 with free unmodified cysteines or 2,827.4 with an intrachain disulfide bond). In the 50-mM DTT map, the 118-min peak was not apparent and a new large peak was observed at 109 min that contained a peptide with a measured mass 3,039.6. This mass matched that of residues 223–249 with 4-vinylpyridine modification of the two cysteines (calculated mass of 3,040.5). The heights of the 118- and 109-min peaks in the two maps were nearly identical.

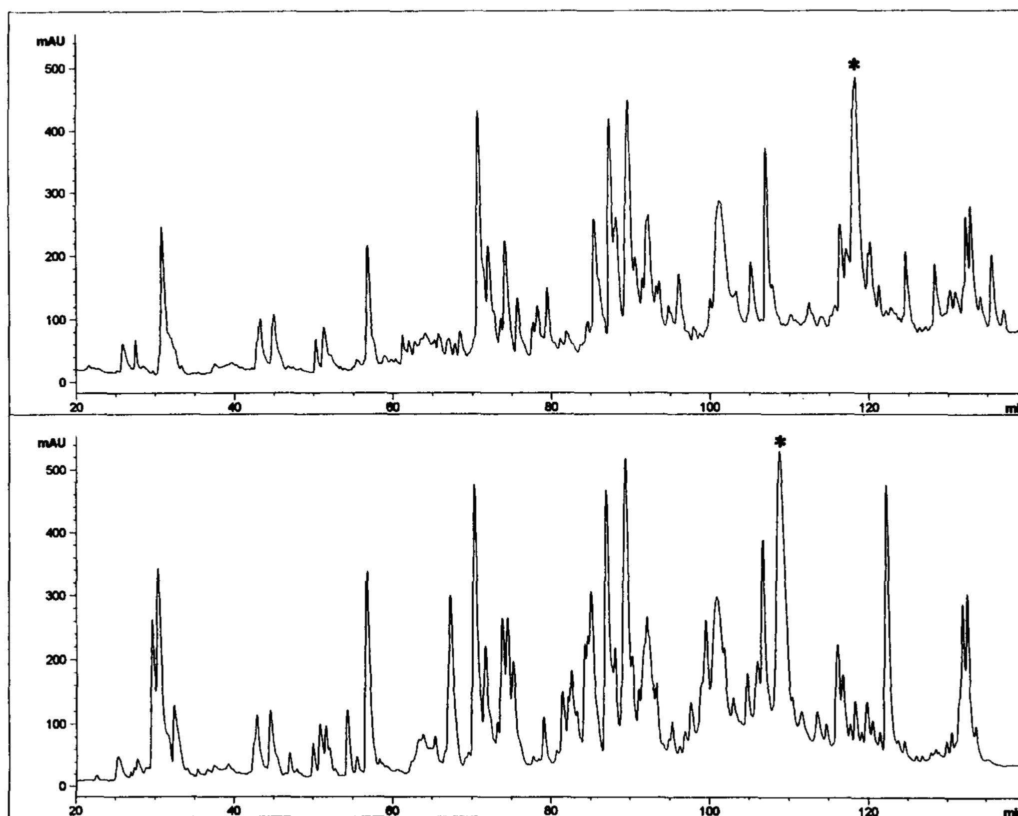

Fig. 3. Identification of an intrachain disulfide bond in the core hinge region of CDP571. Trypsin digest RP-HPLC peptide map chromatograms (210-nm detection) of denatured CDP571 with or without reduction with 50 mM DTT (with 4-vinylpyridine labeling of free cysteines). In the 0-mM DTT map (top), the peak marked with an asterisk contains the heavy chain hinge region peptide YGPPC̲PSC̲PAPEFLGGPSVFLFPPKPK with an intrachain disulfide bond. In the 50-mM DTT map, the peak marked with an asterisk contains the same peptide, but with 4-vinylpyridine alkylation of the two cysteines.

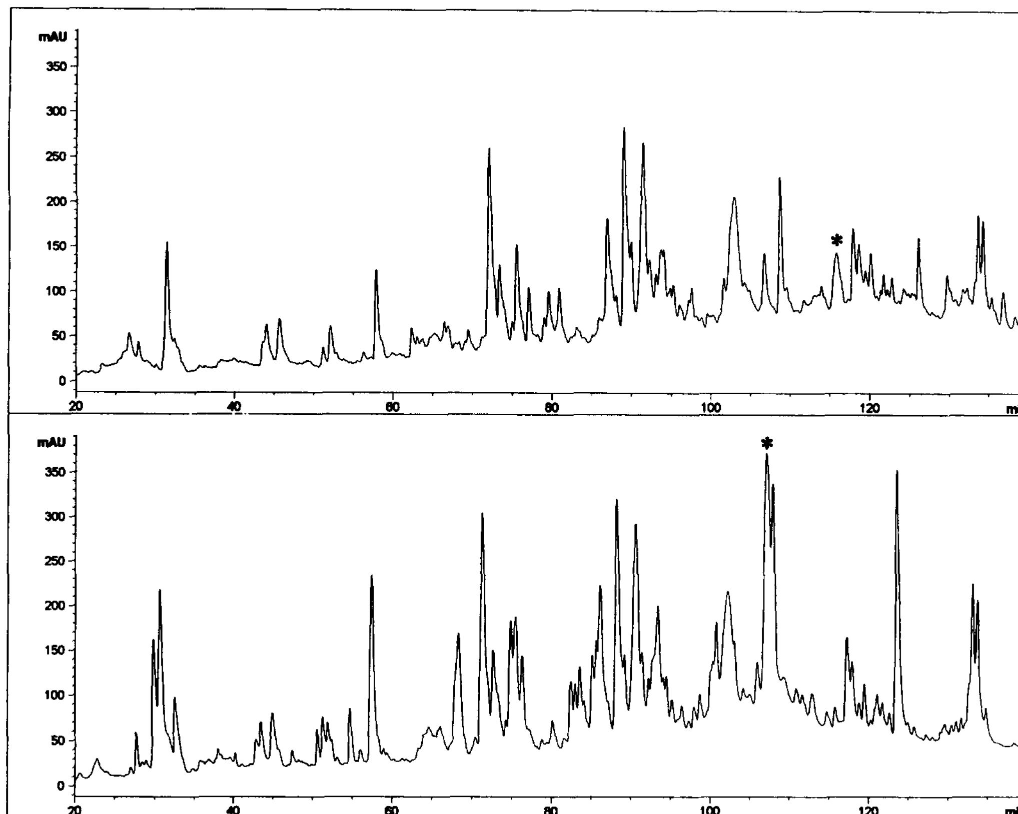

Fig. 4. Identification of an intrachain disulfide bond in the core hinge region of CDP571(γ1). Trypsin digest RP-HPLC peptide map chromatograms (210-nm detection) of denatured CDP571(γ1) with or without reduction with 50 mM DTT (with 4-vinylpyridine labeling of free cysteines). In the 0-mM DTT map (top), the peak marked with an asterisk contains the heavy chain hinge region peptide THTCPPCPAPEFLGGPSVFLFPPKPK with an intrachain disulfide bond. In the 50-mM DTT map, the peak marked with an asterisk contains the same peptide, but with 4-vinylpyridine modification of the two cysteines.

In the 0-mM DTT map of CDP571(γ1) (Fig. 4), a small peak at 116 min was found to contain a peptide with a measured mass of 2,762.2. This mass matched that of the heavy chain hinge region peptide THTCPPCPAPEFLGGPSVFLFPPKPK with an intrachain disulfide bond (residues 228–253, calculated masses of 2,764.4 with free unmodified cysteines or 2,762.4 with an intrachain disulfide bond). The first 11 residues of this peak were sequenced and the primary sequence was found to be THT_PP_PAPE (pyridylethyl-cysteine-phenylthiohydantoin was not observed at residues four and seven), confirming the ESI-MS results. In the 50-mM DTT map, the 116-min peak was not apparent and a new large peak was observed at 107 min that contained a peptide with a measured mass of 2,974.5. This mass matched that of residues 228–253 with 4-vinylpyridine modification of the two cysteines (calculated mass of 2,974.5). The height of the 116-min peak was approximately one-fifth that of the 107-min peak.

The results of this set of experiments demonstrate that the two cysteines in the IgG4 and IgG1 core hinge regions (CPSCP and CPPCP, respectively) are capable of forming an intrachain disulfide bond, thus preventing inter-heavy chain disulfide bond formation between half-IgG molecules.

Reduction and alkylation of native CDP571 and CDP571(γ1)

Trypsin digest RP-HPLC peptide maps of native CDP571 at final DTT concentrations of 50, 5, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015, and 0 mM (with 4-vinylpyridine labeling of free cysteines) were compared, but for reasons of brevity, only the 0-mM and 0.5-mM DTT maps are shown (Fig. 5). Peptides in the labeled peaks in Figure 5 were identified by on-line ESI-MS and, in some cases, Edman sequencing. A summary of these results is given in Table 1. Four peaks, designated as A, B, C, and D in the 0-mM DTT map, were found to decrease in peak height with increasing DTT concentrations. A graph of the relative peak height of Peak C versus DTT concentration is shown in Figure 6. The first 15 residues of Peak A were sequenced and found to be ADYEKH KVYA_EVTH (light chain residues 190–204) and SFNRGE_ (light chain residues 214–220). The measured mass of Peak A was 3,500.8 amu. These data suggest that Peak A contains the light chain peptides 190–213 (ADYEKHKVYACEVTHQGLSSPVTK) and 214–220 disulfide linked through cysteines 200 and 220 (calculated mass of 3,499.6 amu). The first ten residues of Peak B were sequenced and gave VQWKVDNALQ (light chain residues 152–161) and SFN RGEC (light chain residues 214–220 with pyridylethyl-cysteine-phenylthiohydantoin at residue 220). Two measured masses were determined for Peak B: 915.7 and 7,646.4. These data suggest that Peak B contains the light chain peptide 214–220 with cysteine 220 modified with 4-vinylpyridine (916.4 calculated mass) and the light chain peptides 152–213 (VQWKVDNALQSGNSQESVTE QDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPV TK) and 214–220 disulfide linked through cysteines 200 and 220 (calculated mass of 7,644.6). The first ten residues of both Peaks C

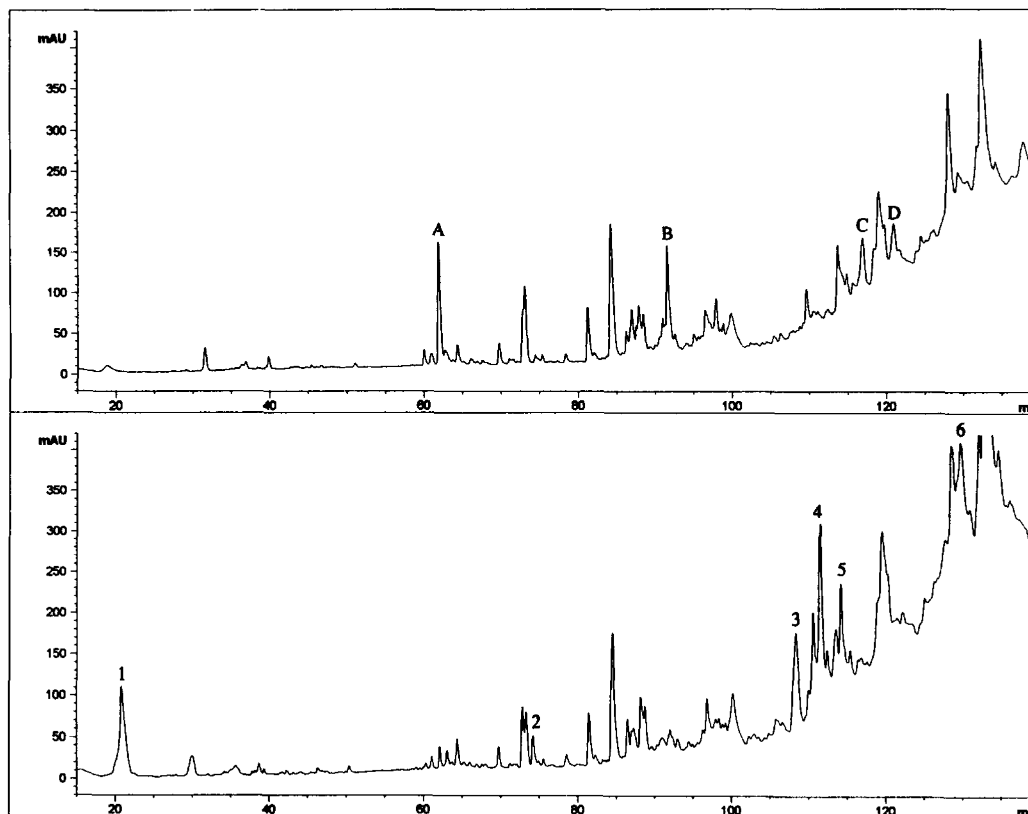

Fig. 5. Reduction and alkylation of native CDP571. Trypsin digest RP-HPLC peptide map chromatograms (210-nm detection) of (top) nonreduced native CDP571 plus 4-vinylpyridine and (bottom) native CDP571 reduced with 0.5 mM DTT and treated with 4-vinylpyridine. Lettered and numbered peaks were analyzed by on-line ESI-MS and, in some cases, collected for Edman sequencing in a second duplicate experiment.

and D were sequenced and found to be YGPP_PS_PA. A comparison of the measured masses with tryptic peptide masses calculated from the sequence of CDP571 indicates that the peaks contain the

Table 1. *Reduction and alkylation of native CDP571*[a]

Peak	Identity
A	Disulfide bonded light chain peptides, residues 190–213 and 214–220
B	Disulfide bonded light chain peptides, residues 152–213 and 214–220 and light chain peptide, residues 214–220 with 4-vinylpyridine modified Cys
C and D	Heavy chain hinge region peptides, residues 223–249 and 223–256, with intrachain disulfide bonds
1	Light chain peptide, residues 214–220 with 4-vinylpyridine modified Cys
2	Heavy chain peptide, residues 126–137 with a modified Cys
3	Heavy chain hinge region peptide, residues 223–249 with two modified Cys
4	Light chain peptide, residues 68–109 with a modified Cys
5	Light chain peptide, residues 68–109 with a modified Cys and heavy chain hinge region peptide, residues 223–256 with two modified Cys
6	Light chain peptide, residues 68–109 with a modified Cys

[a]Identification of labeled peptide map peaks in Figure 5.

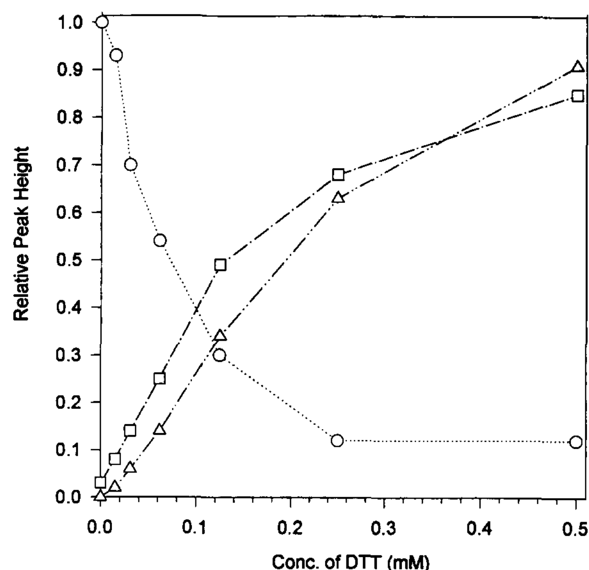

Fig. 6. Graph of the relative peak heights of Figure 5 peaks 1 (△), 3 (□), and C (○) versus DTT concentration. Peak 1, SFNRGEC (4-vinylpyridine modified light chain carboxyl-terminal peptide 214–220). Peak 3, heavy chain hinge region (residues 223–249), YGPPCPSCPAPEFLGGPSVFLF PPKPK, of CDP571 with 4-vinylpyridine labeling of cysteines. Peak C, intrachain disulfide bonded heavy chain hinge region peptides 223–249 and 223–256 of CDP571.

heavy chain hinge region peptides YGPPCPSCPAPEFLGGPSV FLFPPKPK (residues 223–249, 2,828.4 measured mass and calculated masses of 2,829.4 with free unmodified cysteines or 2,827.4 with an intrachain disulfide bond) and YGPPCPSCPAPEFLGGPS VFLFPPKPKDTLMISR (residues 223–256, 3,645.5 measured mass and calculated masses of 3,646.8 with free unmodified cysteines or 3,644.8 with an intrachain disulfide bond), respectively.

In the native CDP571 peptide mapping experiment, six peaks were found to increase in peak height with increasing DTT concentrations (Fig. 5). A graph of the relative peak heights of Peaks 1 and 3 versus DTT concentration is shown in Figure 6. Peak 1 was sequenced and found to be SFNRGEC (light chain residues 214–220). The measured mass of the peptide (916.4) was identical to the calculated mass of the peptide plus the mass of 4-vinylpyridine (916.4). Peak 2 contained a peptide with the sequence GPSVF PLAPCSR, corresponding to heavy chain residues 126–137. The measured mass of the peptide (1,335.3) was the same as that calculated for the peptide plus 4-vinylpyridine (1,334.7). The first 15 residues of Peak 3 were sequenced and found to be YGPPCPSC PAPEFLG. The mass of the peptide (3,040.6) matched that of the heavy chain hinge region of CDP571, residues 223–249 (YGP PCPSCPAPEFLGGPSVFLFPPKPK), with 4-vinylpyridine modification of the two cysteines (calculated mass of 3,040.5). Peak 4 contained two masses (4,342.0 and 4,341.0) that fit light chain residues 68–109 (FIGSGSGTEFTLTISSLQPDDVATYYCQQYY DYPWTFGQGTK) with a modified cysteine (calculated mass of 4,342.2). Peak 5 also contained the light chain residues 68–109 (measured mass of 4,341.0) as well as the mass 3,857.0, corresponding to the heavy chain hinge region residues 223–256 (YG PPCPSCPAPEFLGGPSVFLFPPKPKDTLMISR) with modified cysteines (calculated mass of 3,856.9). Peak 6 contained the mass 4,341.0, which fits light chain residues 68–109.

Figure 6 shows a plot of the relative peak heights of Peak C (intrachain disulfide bonded heavy chain hinge region peptides 223–249 and 223–256), Peak 3 (4-vinylpyridine modified heavy chain hinge region peptide 223–249), and Peak 1 (4-vinylpyridine modified light chain carboxyl-terminal peptide 214–220) versus DTT concentration. The decay of Peak C and rise of Peak 3 with increasing DTT concentration indicates reduction (and concomitant cysteine modification) of the intrachain core hinge region disulfide bond. The SDS-PAGE gel of CDP571 (Fig. 1) suggests that the increase of Peak 3 with DTT concentration is also related to reduction (and cysteine modification) of the two inter-heavy chain disulfide bonds. The sigmoidal nature of the Peak 1 curve is consistent with the SDS-PAGE data in that the inter-heavy chain disulfide bonds are more sensitive to reduction than the heavy–light chain linkage.

Maps of CDP571(γ1) at final DTT concentrations of 50, 5, 1, 0.5, 0.25, 0.125, and 0 mM (with 4-vinylpyridine labeling of free cysteines) were also compared (data not shown). In contrast to the CDP571 experiment, heavy chain hinge region peptides could not be identified in the 0-mM DTT peptide map.

Conformational energy calculations

The results of these studies suggested that the two cysteines in the core hinge region (CPSCP) of CDP571 are far more likely to form an intrachain disulfide bond than those in the core hinge region (CPPCP) of CDP571(S229P) or CDP571(γ1). In order to test this hypothesis, simulated annealing calculations on two cyclic disulfide bonded CPSCP and CPPCP peptides were performed to find the low-energy conformations using the Sybyl program (Tripos, Inc., St. Louis, Missouri) and Kollman force field. The energy ranges for the low-energy conformations were found to be 31–33 kcal/mol for CPSCP and 40–42 kcal/mol for CPPCP. Higher torsion and angle bending energies were observed for the CPPCP peptide due to backbone constraints caused by the extra proline. These modeling results suggest that the serine in the core hinge region of CDP571 allows more hinge region flexibility than the proline of CDP571(S229P) and CDP571(γ1) and thus may permit formation of an intrachain disulfide bond more readily.

Discussion

CDP571 is an engineered human monoclonal antibody with an IgG4 hinge region. This antibody is identical to human myeloma IgG4 and other recombinant monoclonal antibodies with an IgG4 hinge region in that it is secreted as both disulfide bond-linked tetramers and as half-IgG molecules assembled into tetramers by noncovalent interactions (Virella & Parkhouse, 1973; Petersen & Dorrington, 1974; Morrison et al., 1988; Colcher et al., 1989; Tan et al., 1990; Canfield & Morrison, 1991; King et al., 1992; Angal et al., 1993). To identify the chemical factors that prevent covalent association of half IgG4 molecules, CDP571 was genetically engineered to encode proline at residue 229 in the core hinge region rather than serine or with an IgG1 rather than IgG4 hinge region. These mutations of CDP571 conferred upon both antibodies the ability to form disulfide bonds between heavy chains, given that only trace amounts of half-IgG molecules assembled into tetramers by noncovalent interactions were observed. A serine to proline substitution in the core hinge region of a chimeric mouse/human IgG4 (Angal et al., 1993) and the substitution of the hinge sequence in an IgG4 human antibody with that from a human IgG3 antibody (Tan et al., 1990) also resulted in a dramatic reduction of the amount of noncovalently associated half-IgG observed. In agreement with studies on a monoclonal human IgG4 (Virella & Parkhouse, 1973), the inter-heavy chain disulfide linkages of CDP571 were shown to be more sensitive to reduction than the heavy–light chain bridge. Also, in agreement with studies on monoclonal human IgG1, IgG2, and IgG3 (Virella & Parkhouse, 1973), the inter-heavy and heavy–light chain disulfide bonds of CDP571(S229P) and CDP571(γ1) were found to be equally sensitive to reduction.

The stability of small disulfide loops, such as those observed in the core hinge region of CDP571, CDP571(S229P), and CDP571(γ1), have been investigated in model peptides by measuring the thiol-disulfide exchange with glutathione (Weber & Hartter, 1974). Synthetic peptides of the type Cys-glycine$_m$-Cys with $m = 1$, 2, and 3 were found to be very unstable compared with $m = 4$ and 5, probably reflecting bond strain in the disulfide when m is small. A study of the products of air oxidation of the same type of synthetic peptides showed no intramolecular loop formation for $m = 0$ or 1; 15% and 40% loops for $m = 2$ and 3, respectively; and 90% for $m = 4$ (Heaton et al., 1956).

An intrachain disulfide bond between cysteines separated by only two amino acids is not unique to the core hinge region of some IgG molecules. A number of proteins that participate in physiologically important disulfide exchange oxidation/reduction reactions contain active sites with small disulfide loops of the type Cys-X-X-Cys, where X is a non-cysteine residue. These proteins include *Escherichia coli* thioredoxin reductase, Cys-Ala-Thr-Cys (O'Donnell & Williams, 1985); calf thymus and *E. coli* glutaredoxin, Cys-Pro-Tyr-Cys (Hoog et al., 1983; Klintrot et al., 1984);

E. coli and rat liver thioredoxin, Cys-Gly-Pro-Cys (Holmgren et al., 1975; Luthman & Holmgren, 1982); rat liver protein disulfide isomerase, Cys-Gly-His-Cys (Edman et al., 1985); *E. coli* derived DsbA, Cys-Pro-His-Cys (Bardwell et al., 1991; Kamitani et al., 1992; Grauschopf et al., 1995); DsbC, Cys-Gly-Tyr-Cys (Missiakas et al., 1994; Shevchik et al., 1994); and DsbD, Cys-Val-Ala-Cys (Missiakas et al., 1995). The stability of the disulfide bonds in some of these proteins has been determined experimentally. For example, the disulfide bond in the thioredoxin active site was found to be quite stable with an equilibrium constant for forming the disulfide bond (effective concentration) of 10 M using glutathione as a reference species (Lin & Kim, 1989). In contrast, the active disulfides of protein disulfide isomerase are extremely unstable and oxidizing, with a value of 42–60 μM for the equilibrium constant with glutathione (Hawkins et al., 1991; Lyles & Gilbert, 1991), similar to the value measured for DsbA (Zapun et al., 1993).

In summary, peptide mapping studies of CDP571 and CDP571(γ1) identified the chemical factor preventing inter-heavy chain disulfide bond formation between half-IgG molecules: the two cysteines in the IgG4 and IgG1 core hinge regions (CPSCP and CPPCP, respectively) are capable of forming an intrachain disulfide bond. Conformational modeling studies on cyclic disulfide bonded CPSCP and CPPCP peptides suggest a reason why a larger fraction of intrachain bonds are observed in IgG4 rather than IgG1 molecules: the serine in the core hinge region of IgG4 allows more hinge region flexibility than the proline of IgG1 and thus may more readily permit formation of a stable intrachain disulfide bond.

Materials and methods

Reagents

Sequencing grade trypsin was obtained from Promega (Madison, Wisconsin). Dithiothreitol (DTT, 99% pure, #D-0632), 4-vinylpyridine (#V-3877), and iodoacetic acid (#I-4386) were obtained from Sigma (St. Louis, Missouri). Tris-Glycine SDS sample buffer and 4–20% slab gels were purchased from Novex (San Diego, California). The *E. coli* strain CJ236 used in the site-directed mutagenesis procedure was obtained from Invitrogen (San Diego, California) [genotype: *F′cat* {= pCJ105; M13^{s}Cam^{r}}/*dut ung1 thi-1 rel*A1 *spo*T1 *mcr*A]. All DNA modification enzymes were purchased from New England Biolabs (Beverly, Massachusetts).

Mutagenesis

The 1,959-bp *Sal* 1 restriction fragment from vector pAHDG571 (Celltech Therapeutics Ltd., Slough, Berks., UK) was subcloned into a pUC118 phagemid to make the mutagenesis template plasmid pUC.G_4Hc. This insert contained most of the constant region from the IgG4 heavy chain gene. Site-directed mutagenesis was done by the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987). The serine 229 residue of the IgG4 heavy chain hinge was changed to a proline residue using 5′-C-AAA-TAT-GGG-CCC-CCA-TGC-CCA-CCA-TGC-CCA-G-3′ as the mutagenesis primer. Positive clones were identified by the presence of a unique *Apa* 1 site introduced by the mutagenesis primer. The S229P mutation was confirmed by sequencing the DNA from a positive clone and the modified heavy chain gene fragment was transferred back into the parental vector to make the pAHDG571(S229P) vector.

The pAHDG571(γ1) vector was constructed in two steps. An *Xba* 1 site was introduced at the 3′ end of the IgG4 hinge in vector pUC.G_4Hc by site-directed mutagenesis using 5′-GC-CCA-GGT-AAG-CCA-TCTAGA-GGC-CTC-GCC-3′ as the synthetic primer. Next, the IgG4 isotype hinge domain was removed by cutting with *Pst* 1 and *Xba* 1, and replaced with a double-stranded synthetic DNA insert coding for the IgG1 isotype hinge. The new modifications were confirmed by DNA sequencing and the pUC insert was transferred into the parental pAHDG571 vector to make pAHDG571(γ1).

Transfection and cell culture

Chinese hamster ovary cells (CHO-AA8) were transfected with either pAHDG571(S229P) or pAHDG571(γ1) expression vectors using Lipofectin (GibcoBRL, Grand Island, New York). Stable cell lines were obtained by single-cell cloning in L-methionine sulfoximine selection medium (Sigma, St. Louis, Missouri).

Protein purification

CDP571, CDP571(S229P), and CDP571(γ1) were purified from tissue culture fluid by application of the sterile filtered solution to a Protein A Hi Capacity resin (PerSeptive Biosystems, Cambridge, Massachusetts) column (1.6 × 5.0 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with 10 column volumes of equilibration buffer. The antibody was eluted from Protein A with 0.1 M glycine, pH 2.7, and neutralized with 1/10 volume of 1.0 M Tris buffer, pH 8.0. IgG-containing fractions were pooled and concentrated using an Amicon YM 30 membrane (Amicon, Inc., Beverly, Massachusetts) and dialyzed against phosphate buffered saline (PBS), pH 7.2. The dialyzed antibody solution was then sterile filtered and frozen at −70 °C.

SDS-PAGE

One milligram of antibody was lyophilized and reconstituted in 500 μL of PBS prior to division into eight 50-μL aliquots with final DTT concentrations of 5, 1, 0.5, 0.25, 0.125, 0.062, 0.031, and 0.015 mM. The samples were incubated at 37 °C for 1 h. A 3-μL volume of 2 M iodoacetic acid was then added and the samples were incubated at room temperature in the dark for 30 min. A 15-μL volume was removed from each tube, mixed with the same volume of 2× Tris-glycine SDS sample buffer, and incubated for 5 min at 100 °C. A 10-μL aliquot (10 μg of protein) of each sample was then added per lane of the slab gel. Gels were stained with Fast Stain (Zoion Biotech, Newton, Massachusetts). Densitometry analysis of the gels was performed using a Molecular Dynamics (Sunnyvale, California) Personal Densitometer SI with Softmax analysis software.

Enzyme digestion

Antibody sequence confirmation was performed as follows. A 1-mg lyophilized antibody sample was solubilized in 500 μL of 6 M guanidine hydrochloride, 0.05 M Tris, pH 8.6. A 12.5-μL volume of 2 M DTT was added and the sample was heated at 100 °C for 1 h. A 30-μL volume of 2 M iodoacetic acid was added and the sample was incubated in the dark for 30 min. The samples were dialyzed extensively against 0.1 M NH_4HCO_3 (pH not adjusted), then digested by adding 10 μg of trypsin to each 1 mg sample of

antibody and incubating at 37 °C for 18 h. Reactions were stopped by heating at 100 °C for 5 min.

Peptide mapping

Reduction and alkylation of native CDP571 and CDP571(γ1)

A 2-mg/mL solution of CDP571 or CDP571(γ1) in PBS was divided into ten 1-mL aliquots with final DTT concentrations of 50, 5, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015, and 0 mM. The tubes were incubated at 37 °C for 1 h. The samples were cooled to room temperature and 8 μL of 4-vinylpyridine was added to each tube. The samples were then incubated for 90 min at room temperature in the dark. Each sample was exhaustively dialyzed against PBS and lyophilized. The lyophilized samples were then reconstituted in 330 μL of 6 M guanidine-HCl in 0.1 M NH_4HCO_3 (pH not adjusted) and heated for 2 h at 70 °C. After cooling, 670 μL of 0.1 M NH_4HCO_3 was added to each tube and the samples were digested by adding 20 μg of trypsin and incubating at 37 °C for 18 h. The reaction was stopped by heating at 100 °C for 5 min.

Reduction and alkylation of denatured CDP571 and CDP571(γ1)

One hundred microliter aliquots of a 20-mg/mL solution of CDP571 or CDP571(γ1) were added to two tubes containing 900 μL of 6 M guanidine-HCl in 0.1 M NH_4HCO_3. A 25-μL volume of 2 M DTT was then added to one tube and a 25-μL volume of 6 M guanidine-HCl was added to a control tube. The tubes were then incubated at 100 °C for 1 h. The samples were cooled to room temperature and 8 μL of 4-vinylpyridine was added to each tube. The samples were then incubated for 90 min at room temperature in the dark, then dialyzed extensively against 0.1 M NH_4HCO_3 (pH not adjusted), and digested by adding 10 μg of trypsin to each 1 mg sample of antibody and incubating at 37 °C for 18 h. The reaction was stopped by heating at 100 °C for 5 min.

HPLC

Trypsin-digested antibody samples (approximately 200 μg per injection) were chromatographed by RP-HPLC using a Hewlett Packard 1090 Series M system with an integrated diode-array detector. Hewlett Packard Chemstation Rev. A. 02.00 software was used for data analysis. A Vydac 218TP52 (C18, 5-μm, 250 × 2.1 mm I.D.) column was used with the following chromatographic conditions: flow rate, 0.15 mL/min; oven temperature, 40 °C; detection 210, 254, 280, and 295 nm; solvent A, 0.1% TFA; solvent B, 0.1% TFA–60% acetonitrile; gradient, 0 min 10% B; 5 min 10% B; 140 min 65% B; 145 min 100% B; 150 min 10% B at 0.30 mL/min; 165 min 10% B.

ESI-MS

The HP1090 HPLC column effluent, at a flow rate of 0.15 mL/min, was mixed with 40% propionic acid/isopropyl alcohol (0.15 mL/min from an HP 1050 HPLC pump) and introduced directly into a Hewlett Packard model 59987A API-Electrospray LC/MS Interface with a model 5989B MS Engine. The quadrupole was scanned from 450 to 2,000 m/z in 2 s in the positive ion mode. Conditions used for data acquisition were: integration time, 100 μs; samples, 4; step size, 0.1 amu; mass response filter, Gaussian FWHM 0.300 amu; and time response filter, Gaussian FWHM 0.050 min. The instrument was tuned with a mixture of Cytochrome *c* and Gramicidin S at a peak width of approximately 1.2 amu. Data analysis was accomplished with an HP ChemStation data system with HP G1047A LC/MS Software. Most abundant ion peptide molecular masses were calculated from the amino acid sequence using HP Peptide Tools software.

Sequence analysis

Peptide map peaks requiring N-terminal sequence analysis for identification were collected manually in a duplicate chromatographic experiment and stored frozen at −70 °C. Automated Edman degradation was performed with an Applied Biosystems model 473A Pulsed Liquid protein sequencer system and data were analyzed with the model 610A program.

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