Hierarchical Formation of Disulfide Bonds in the Immunoglobulin Fc Fragment Is Assisted by Protein-disulfide Isomerase*

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Antibodies provide an excellent system to study the folding and assembly of all β-sheet proteins and to elucidate the hierarchy of intra/inter chain disulfide bonds formation during the folding process of multimeric and multidomain proteins. Here, the folding process of the Fc fragment of the heavy chain of the antibody MAK33 was investigated. The Fc fragment consists of the CH3 and CH2 domains of the immunoglobulin heavy chain, both containing a single S-S bond. The folding process was investigated both in the absence and presence of the folding catalyst protein-disulfide isomerase (PDI), monitoring the evolution of intermediates by electrospray mass spectrometry. Moreover, the disulfide bonds present at different times in the folding mixture were identified by mass mapping to determine the hierarchy of disulfide bond formation. The analysis of the uncatalyzed folding showed that the species containing one intramolecular disulfide predominated throughout the entire process, whereas the fully oxidized Fc fragment never accumulated in significant amounts. This result suggests the presence of a kinetic trap during the Fc folding, preventing the one-disulfide-containing species (1S2H) to reach the fully oxidized protein (2S). The assignment of disulfide bonds revealed that 1S2H is a homogeneous species characterized by the presence of a single disulfide bond (Cys-130–Cys-188) belonging to the CH3 domain. When the folding experiments were carried out in the presence of PDI, the completely oxidized species accumulated and predominated at later stages of the process. This species contained the two native S-S bonds of the Fc protein. Our results indicate that the two domains of the Fc fragment fold independently, with a precise hierarchy of disulfide formation in which the disulfide bond, especially, of the CH2 domain requires catalysis by PDI.

Protein folding of disulfide-containing proteins has been studied essentially using small monomeric, one-domain proteins as a model (1–7). The analysis of the folding process of multimeric or multidomains proteins containing intra- and interchain disulfide bonds is hampered by several factors including the presence of an increasing number of folding intermediates and the superimposition of different folding events. Antibodies are multimeric proteins consisting of different domains characterized by two antiparallel β-sheets linked by an intradomain disulfide bond (8–10). A characteristic feature of antibodies is that the intradomain disulfide bond, which connects residues far apart in sequence, is completely buried in the core of the protein. These proteins provide an excellent system to study the folding and assembly of all β-sheet proteins and to elucidate the hierarchy of intra/interchain disulfide bond formation during the folding process of multimeric and multidomain proteins (11–15). The β-sheet folding is usually much slower than the α-helix formation because amino acid residues, which are far apart in the polypeptide chain, must interact correctly in the three-dimensional space to form stabilizing interactions (16).

Mechanistic studies on oxidative folding of immunoglobulins were previously carried out on antibody fragments and single antibody domains (11–13, 15, 17). Among these, the simplest model system is the non-covalent homodimer formed by the C-terminal domain C1μ, containing a single intramolecular disulfide bond. The influence of this disulfide on the structure and stability of C1μ has been described (18). It has been shown that structure formation is required to bring the cysteines into proximity to form the disulfide bond and to shield it from the solvent. Conversely, some degree of structural flexibility is required for redox shuffling and rearrangements (18).

In vivo, native disulfide bond formation can occur via multiple parallel pathways, and there is evidence that a large number of different gene families and redox carriers may play a role in supplying redox equivalents for protein disulfide bond formation (19–21). The rate-limiting steps for native disulfide bond formation in proteins like antibodies, which contain multiple disulfides, are late-stage isomerization reactions, where disulfide bond formation is linked to conformational changes in protein substrates. These steps are catalyzed by proteins belonging to the protein-disulfide isomerase (PDI) family, which accelerate formation and reshuffling of disulfide bonds until the native structure is resistant to further rearrangements (22). It has been shown that in vitro PDI promotes the correct

* The work was supported by European Community Grant BIO4-CT96-0436 and Progetto Finalizzato Consiglio Nazionale delle Ricerche, “Folding of recombinant immunoglobulins”, FIRB 2002 “Folding di proteine, l’altra meta del codice genetico”, and MIUR_Rome PRIN 2001-COFINLAB2001. The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereon marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PDI, protein-disulfide isomerase; ESIMS, electrospray ionization mass spectrometry; GdmCl, guanidine chloride; GSSG, oxidized glutathione; MALDIMS, matrix-assisted laser desorption ionization mass spectrometry; HPLC, high performance liquid chromatography.
disulfide bond formation in a Fab fragment. Interestingly, the accessibility of the cysteine residues during the folding process seems to be of crucial importance for the efficiency of PDI (13). The endoplasmic reticulum-specific chaperone immunoglobulin heavy chain-binding protein (BiP) acts synergistically with PDI in assisting the folding of the denatured and reduced Fab fragment (23). BiP binds the unfolded immunoglobulin chains and keeps them in a conformation in which cysteine residues are accessible to PDI.

Here the folding process of the recombinantly produced Fc fragment of the heavy chain of the murine monoclonal antibody MAK33 directed against the muscle-specific isoform of human creatine kinase was investigated in the absence and in the presence of PDI. The Fc fragment consists of the C1r3 and C1r2 domains of the immunoglobulin heavy chain, both containing a single S-S bond. The evolution and the relative abundance of intermediates in the folding of the Fc fragment were monitored by electrospray mass spectrometry. The specific disulfide bonds present in the folding mixture at different times were assessed by mass mapping to determine the pathway of disulfide bonds formation in the presence or absence of PDI. The results obtained indicate a precise hierarchy in the formation of disulfide bonds during the folding process and underline the key role exerted by PDI in the folding mechanism of the Fc fragment.

**EXPERIMENTAL PROCEDURES**

Reduced dithiothreitol, EDTA, reduced glutathione, and oxidized glutathione were obtained from Sigma; Tris and iodoacetamide were purchased from Fluka. Endoproteinasin Asp-N was acquired from Roche Applied Science. Guanidinium chloride (GdmCl) and cyanogen bromide were from Pierce. All other reagents were of the highest grade commercially available. PDI was purified as described (23).

**Expression of Fc Fragment—**The Fc fragment of MAK33 was cloned into a pQE-based expression vector by standard techniques. For expression, the Escherichia coli strain HB 101 (24) containing the repressor plasmid pUBS520 (25) was transformed with the plasmid. The strain was grown in SB ampicillin/kanamycin medium at 37°C and induced with 1.5 mM isopropyl-1-thio-β-D-galactopyranoside at an Amax of 0.8–1.0. Cells were harvested by centrifugation 20 h later and resuspended in 100 mM Tris/HCl, 1 mM EDTA, pH 7, at 4°C (5 ml of wet cells). After lysis in a BasicZ cell disruptor (Constant Systems, Warwick, UK), the soluble fraction was separated from the insoluble fraction by centrifugation at 48,000 × g for 30 min. The Fc fragment was found in the insoluble fraction as inclusion bodies.

Inclusion bodies were further purified according to standard procedures (Rudolph et al. (26)). The yield of inclusion bodies was 17% of the wet cell mass. The inclusion body protein was solubilized in 100 mM Tris/HCl, 6 M GdmCl, 100 mM dithioerythritol, 1 mM EDTA, pH 8, for 2 h at 25°C. Then the pH value was shifted to pH 2–3, and the solution was centrifuged at 48,000 × g and dialyzed overnight against 4 M GdmCl, pH 2, at 4°C. The protein concentration was determined as described (27).

Starting from established procedures for antibody refolding (17, 28), refolding and disulfide bond formation of Fc were optimized. In the final protocol, 4 times 80 μg/ml denatured and reduced Fc were added to the refolding buffer, consisting of 100 mM Tris/HCl, 0.5 mM t-ariginine, 1 mM GSSG, 1 mM GSH, pH 7.5, at 10°C. The time between the additions was 2 h. After 150 h, 1.5 mM ammonium thiocyanate was added to the renaturation solution followed by centrifugation at 48,000 × g for 1 h. The supernatant was loaded on a butyl-Sepharose column equilibrated with 100 mM Tris/HCl, 2 mM EDTA, 1.5 mM ammonium thiocyanate, pH 7. Elution was achieved with a linear gradient from 1.5 to 0 mM ammonium sulfate. The Fc-containing fractions were determined by SDS-PAGE, pooled, and applied to a Sephadex 75-pg gel filtration column (Amersham Bioscience) equilibrated with 100 mM Tris/HCl, 2 mM EDTA, 200 mM NaCl, pH 7. The Fc-containing fractions were dialyzed against 50 mM phosphate buffer, pH 7.5, and frozen in aliquots. The purity was confirmed by silver-stained SDS-PAGE. Fc concentrations were determined photometrically (A280 nm = 1.33).

The Fc fragment preparation was further purified by reverse phase HPLC using a Phenomenex C4 column (5 μm, 1 × 25 cm). The elution system consisted of 0.1% trifluoroacetic acid (solvent A) and 0.07% trifluoroacetic acid in acetonitrile/2-propanol (2:1 v/v) (solvent B) with a linear gradient of solvent B from 35 to 95% at a flow rate of 3 ml/min. The elution was monitored at 220 nm. The intact Fc fragment was recovered and lyophilized. The lyophilized protein was then analyzed by ESIMS.

**Spectroscopic Characterization of Fc—**For spectroscopic characterization, samples were dialyzed against 50 mM NaHPO4/Na2HPO4, pH 7.5, 200 mM NaCl. For denaturation of the oxidized Fc fragment, samples were incubated for 12 h at 20°C in 50 mM NaHPO4/Na2HPO4, pH 7.5, 200 mM NaCl, 6 M GdmCl, 1 mM dithiothreitol.

The intrinsic fluorescence of the Fc fragment was measured in a Fluoromax-2 (Spx, Edison-NJ) fluorimeter at 20°C. Emission spectra were recorded in the range of 290–440 nm with an excitation wavelength of 290 nm and a scanning rate of 5 nm. The spectral bandwidth was 3 nm for excitation and 8 nm for emission.

Far UV-circular dichroism spectra were recorded at 20°C in a Jasco J-715 spectropolarimeter (Jasco, Grossumstadt, Germany) with a PTC 343 Peltier unit using a quartz cell with a path length of 1 mm. The spectra were scanned at 20 nm/min over a wavelength range of 195–260 nm. Spectra were corrected for contributions from the buffer.

**Analytical Gel Filtration—**Analysis of folded Fc fragment by size exclusion chromatography HPLC was performed on a PU-1580 system (Jasco) a using Superdex 75 HR column (Amersham Biosciences). The buffer used was 40 mM Hepes, 150 mM KCl, pH 7.5. Protein fluorescence was detected using a FP 1520-S fluorescence detector (Jasco) with an excitation wavelength of 280 nm and emission at 343 nm. The flow rate was 0.5 mm/min. The standard protein used were ribonuclease A, chymotrypsogenin A, ovalbumin, and albumin (Amersham Biosciences).

**Folding Reactions—**Lyophilized Fc fragment was dissolved in water, and the protein concentration was determined according to Bradford (27) using Fab fragment as the standard.

The Fc fragment was reduced and denatured in 4 M GdmCl, 0.1 M Tris, pH 8.0, 1 mM EDTA, 300 mM dithiothreitol at a final protein concentration of 5 mg/ml for 90 min at room temperature under a nitrogen atmosphere. Folding was initiated by diluting the denatured protein 100-fold with vigorous stirring for 10 s into the buffer preincubated at 4°C containing 1 mM t-ariginine, 0.1 mM Tris-HCl, pH 8.0, 5 mM EDTA, 6 mM GSSG. The initial concentration of GSSG of the folding was, therefore, 50 μM (2.1 μm). The reaction was carried out at 4°C under nitrogen atmosphere. When the folding was carried out in the presence of PDI, the enzyme was preincubated in the folding buffer containing 1 mM t-arginine, 0.1 mM Tris-HCl, pH 8.0, 5 mM EDTA, 6 mM GSSG for 10 min at 4°C. The final PDI concentration in the folding experiment was 0.4 μM.

**Alyklation of Folding Aliquots—**Aliquots of the folding mixture, sampled at appropriate intervals, were alkylated as described (18, 29). Iodoacetamide was freshly dissolved in 1 M Tris HCl, pH 8.0, containing 10 mM EDTA. During the preparation of the reagents, the solution was protected from light to minimize photolytic production of iodine, a potential oxidizing agent for thiol. The volume was added to an equal volume of a 1 M iodoacetamide solution. Alkylation was performed for 10 s in the dark at room temperature under a nitrogen atmosphere. After 10 s 100 μl of 20% trifluoroacetic acid were added, and the samples were desalted by reverse phase HPLC using a Vydac C4 column (5 μm, 0.46 × 25 cm). The solvent system consisted of 0.1% trifluoroacetic acid (solvent A) and 0.07% trifluoroacetic acid in acetonitrile/2-propanol (2:1 v/v) (solvent B). Folding intermediates were eluted with a linear gradient of solvent B from 20 to 95% at a flow rate of 1 ml/min. Eluted proteins were monitored at 220 nm, recovered, directly analyzed by ESIMS, and then lyophilized for subsequent analysis.

**Identification of Disulfide Bonds in the Folding Intermediates—**Carboxymidomethylated folding intermediates were hydrolyzed with cyanogen bromide and then protozotically digested with endoproteinasin Asp-N. Cyanogen bromide hydrolysis was carried out in 200 μl of 70% trifluoroacetic acid using a 10-fold molar excess of reagent on methionine residues for 18 h at room temperature in the dark. The cyanogen bromide reaction was stopped by adding 10 volumes of cold water and lyophilizing the sample. An aliquot was analyzed by MALDIMS. The rest of the sample was resuspended in water and lyophilized several times. Digestion with endoproteinasin Asp-N was then performed on the mixture of peptides obtained from the cyanogen bromide. Asp-N hydrolysis was carried out in 0.8% ammonium bicarbonate, pH 8.0, using 10% acetonitrile as the activator at 37°C for 18 h with an enzyme-substrate ratio of 1:100 (w/w).

**Electrospray Mass Analyses—**Protein samples were analyzed by ESIMS using a Bio-Q triple quadruple mass spectrometer equipped with an electrospray ion source (Micromass, Manchester, UK) or by...
RESULTS

Characterization of the Fc Fragment—The Fc fragment of MAK33 was expressed in insoluble form in the cytoplasm of E. coli. The protein was refolded and purified to homogeneity. Analytical gel filtration in combination with SDS-PAGE revealed the presence of a noncovalent dimer with an apparent mass of 44 kDa.

The Fc fragment was characterized by far UV-circular dichroism spectroscopy. As shown in Fig. 1A, the protein exhibits a spectrum typical for an immunoglobulin domain. The low negative ellipticity of $-2500$ to $-3000$ $\Phi$ with minima at 218 and 229 nm suggest that the protein is $\beta$-structured. The minimum at 229 nm may result from the contribution of aromatic amino acids to the CD signal as previously observed for the isolated C1$\beta$3 domain (18). As expected, the denatured and reduced protein did not exhibit a CD spectrum with specific minima.

The fluorescence spectra of the denatured and reduced Fc fragment show that the fluorescence is quenched in the native state (Fig. 1B). This is a typical feature of antibody domains that results from the positioning of a conserved Trp residue next to the disulfide bond. The shift of the wavelength of maximum fluorescence from 343 to 354 nm upon unfolding and reduction shows that the buried Trp becomes solvent-exposed.

Taken together, the spectroscopic data show that Fc is natively folded and oxidized.

The recombinant Fc fragment was further purified by reverse phase HPLC just before folding experiments. The HPLC peak corresponding to the Fc fragment was tested by ESI-MS. The analysis revealed the presence of a single component exhibiting a molecular mass of 23885.2 $\pm$ 2.3 Da, in agreement with the expected value, calculated on the basis of its amino acid sequence (23885.0 Da). The purified samples were then used in the folding experiments.

Oxidative Folding of the Fc Fragment—Folding of the Fc fragment from the fully denatured and reduced protein to the native state was carried out at a protein concentration of 50 $\mu$g/ml in the presence of a low concentration of guanidinium chloride (40 mM) and 1 mM l-arginine, a "labilizing" agent that preferentially destabilizes incorrectly folded or aggregation-prone species (28, 30). Aliquots of the folding reactions were withdrawn at different time points, and the intermediates present in solution were trapped by alklylation of the free thiol groups and analyzed by ESI-MS to identify the disulfide-bonded species formed. The carboxamidomethylation reaction used to trap the free SH group increased the molecular mass of the intermediates by a fixed amount, 57 Da for each free SH group, thus allowing the separation by mass of intermediates containing different numbers of disulfide bonds. In addition, ESIMS analysis allowed the determination of the relative abundance of folding intermediates provided that the different components have comparable ionization properties (18, 31).

Fig. 2A shows the electrospray mass spectrum of the folding mixture after 15 s of incubation in the refolding buffer. Each population of trapped intermediates is characterized by a different number of intramolecular disulfide bonds (indicated as nS), mixed disulfides with the exogenous glutathione (nG), and carboxamidomethyl groups. The number of carboxamidomethyl groups corresponds to the number of free thiolis present in the folding intermediates and is, therefore, indicated as nH throughout the text. The spectrum revealed the presence of a major component corresponding to the fully reduced species, 4H. A minor protein species carrying a molecule of exogenous glutathione and three free cysteine residues (1G3H) was also detected in the spectrum.

The distribution of charged states in electrospray analyses is related to the number and accessibility of ionizable groups on the protein surface (32–34). The 4H species showed a distribution of multiple charges with signals centered from 21 to 25
positive charges. The distribution of multiply charged ions displayed by the 1G3H species was similar to that observed for the reduced protein, suggesting a similar open and flexible conformation for this intermediate.

The electrospray spectra were acquired at different times of folding reaction and showed the evolution of the intermediates containing different numbers of disulfide bonds. The relative percentage of the different folding intermediates was plotted against time of incubation in Fig. 3A. The reduced species (4H) decreased to a 20% level after 30 min of incubation and slowly disappeared within 5 h. The species containing one intramolecular disulfide and two free cysteines (1S2H) rapidly appeared in the first stages of the reaction and predominated over the whole process. Under these conditions the fully oxidized species (2S) formed only at very low amounts, never reaching levels higher than 15% during the entire folding process. Accordingly, the intermediate containing one S-S bond and one mixed disulfide with glutathione (1S1G1H, the obligatory precursor of the fully oxidized species 2S) was only detected at very low amounts, never reaching levels higher than 15% during the entire folding process. Accordingly, the intermediate containing one S-S bond and one mixed disulfide with glutathione (1S1G1H, the obligatory precursor of the fully oxidized species 2S) was only detected at very low amounts, never reaching levels higher than 15% during the entire folding process.

Oxidative Folding of the Fc Fragment in the Presence of PDI—To test whether PDI had an influence on the oxidation pathway, the folding of the Fc fragment was carried out in the presence of the catalyst. Fig. 2B shows the electrospray mass spectrum of the intermediates mixture after 24 h of incubation in the presence of PDI. The signals of the fully oxidized species dominated the spectrum, whereas the one disulfide-containing intermediate (1S2H) was detected as a minor component. The 2S species showed a distribution of multiply charged signals ranging from 14 to 19 positive charges. This distribution is quite different from that observed for both the 4H and 1G3H species (see Fig. 2A), and the lower number of positive charges suggests the occurrence of a more compact and tight conformation for the 2S species. The 1S2H species showed a distribution of multiply charged ions ranging from 19 to 23 positive charges that resembles the charge state detected for the reduced protein. This result suggests that the 1S2H intermediate still has an open and flexible conformation.

Fig. 3B shows the time-course analyses of the catalyzed process. A catalytic effect of PDI was clearly detected throughout the entire process. The reduced protein 4H disappeared within 30 min, and the 1S2H species predominated only up to about 5 h when the fully oxidized protein 2S started to accumulate. The 2S species appeared in the early stages of the process and reached about a 90% level after 24 h. This rapid formation of the 2S species in the presence of the enzyme was the most striking difference between the uncatalyzed and the PDI-catalyzed process.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Multiply charged ion atmospheric pressure ionization mass spectra of samples from an uncatalyzed folding reaction withdrawn after 15 s of incubation (A) and from the PDI-assisted folding reaction withdrawn after 24 h of incubation (B). Each peak is composed by multiple lines due to the presence of Na⁺ and K⁺ adducts. Each population of trapped intermediates is characterized by a different number of intramolecular disulfide bonds (indicated as nS), mixed disulfides with the exogenous glutathione (nG), and carboxyamidomethyl groups. The number of carboxyamidomethyl groups corresponds to the number of free thiols present in the folding intermediates and are, therefore, indicated as nH in the figure. 4H, reduced protein; 1G3H, species containing one mixed disulfide with glutathione and three free cysteines; 1S2H, species with one intramolecular disulfide; 2S, oxidized protein.
Identification of Disulfide Bonds in the Uncatalyzed Folding of the Fc Fragment—Aliquots of the uncatalyzed folding mixture of the Fc fragment were sampled at 15 s, 90 min, 5 h, and 24 h of incubation and alkylated as described. To identify the S-S bonds present in the folding mixtures, the aliquots were chemically hydrolyzed with cyanogen bromide, and the resulting peptide mixtures were directly analyzed by MALDIMS. As an example, Fig. 4A shows the MALDI spectrum of the CNBr peptides from the sample withdrawn at 90 min. The signal at \( m/z \) 6533.5 was assigned to the peptide 122–131 linked to the C-terminal fragment 162–210 by the S-S bridge between Cys-130 and Cys-188. This disulfide bond is the native linkage occurring in the CH3 domain.

Moreover, Cys-188 was found involved in a mixed disulfide with glutathione, as demonstrated by the signal at \( m/z \) 5792.2 corresponding to the peptide 162–210, linked to a GSH moiety. In addition, the spectrum showed the presence of a signal at \( m/z \) 5573.1 that was assigned to the peptide 73–121 containing the carboxyamidoterminal Cys-84. Finally, the signal at \( m/z \) 5545.9 corresponded to the peptide 162–210, with Cys-188 alkylated by iodoacetamide.

Table I reports the S-S bonds identified in the aliquots withdrawn at different times. After 15 s of refolding, the MALDI analysis showed only the signal at \( m/z \) 5792.6, corresponding to the mixed disulfide between Cys-188 and glutathione, thus indicating that formation of this derivative constitutes the initial step in the oxidative folding of Fc fragment. After 90 min of folding, mass spectral analysis identified the presence of the Cys-130–Cys-188 S-S bond, corresponding to the native linkage of the CH3 domain, as outlined above. This disulfide was the only intramolecular S-S bond detected up to late stages of reaction and was still present after 24 h of folding. These results confirmed that the 1S2H species constitutes the major intermediate in the folding process as previously shown by kinetic analysis (Fig. 3A). Moreover, the assignment of S-S bonds in the folding intermediates demonstrated that 1S2H is a homogenous species only containing the CH3 intradomain disulfide.

Finally, the MALDI spectrum of the peptide mixture from the aliquots withdrawn at late stages of the process revealed the presence of a weak signal at \( m/z \) 13701.5 assigned to the peptides 1–72 and 73–121 linked to the Cys-24–Cys-84 S-S bridge. This disulfide corresponds to the native intradomain linkage occurring in the CH2 domain, and its presence accounts for the minute amount of the fully oxidized protein observed in the kinetic analysis (Fig. 2A).

The assignment of disulfides were confirmed by proteolytic subdigestion of the CNBr peptide mixtures with endoproteinase Asp-N that shifted the mass signals to lower mass values by removal of short peptides. The signal at \( m/z \) 5792.2, corresponding to the peptide 162–210 with Cys-188 linked to the exogenous glutathione, was shifted to \( m/z \) 2510.1, corresponding to the peptide 181–200 linked to glutathione. The signal at
The strategy described above was employed to assign the glutathione at the initial stages of the process. $m/z$ 6533.5 moved down to $m/z$ 6354.3, corresponding to the peptide pair 124–131 and 162–210 linked by the Cys-130–Cys-188 S-S bridge. Two new signals were detected in the spectra at 90 min and 5 h, at $m/z$ 3794.4 and 3679.9. These peaks were assigned to the peptides (1–32) and (1–31), linked to glutathione, respectively (theoretical values, 3794.3 and 3679.2). Thus, Cys-24 in the $\alpha 1$2 domain does not form a mixed disulfide with glutathione at the initial stages of the process.

Identification of Disulfide Bonds in the PDI-assisted Folding—The strategy described above was employed to assign the disulfide bonds occurring in the folding intermediates of the PDI-assisted reaction. The high mass region of the MALDI spectrum of the CNBr fragments from the aliquot withdrawn at 24 h is shown in Fig. 4B. Two intense mass signals were detected at $m/z$ 13701.9 and 10158.9. The former was assigned to the peptide pair 1–72 and 73–121 linked by the S-S bridge Cys-24–Cys-84, whereas the second peak was interpreted as arising from the 122–210 peptide containing the intramolecular S-S bond, Cys-130–Cys-188. The low mass region of the spectrum confirmed the presence of this S-S bond throughout the signal at $m/z$ 6533.8, corresponding to the peptides 122–131 and 162–210, linked by the Cys-130–Cys-188 disulfide (data not shown).

The mass mapping analysis reported in Table II demonstrated that both native disulfides of Fc fragment already occurred at the early stages of the reaction in the presence of PDI. Non-native S-S bonds were never observed throughout the entire process. Moreover, the assignment of disulfide bonds of the folding intermediates formed after 30 min demonstrated that only Cys-24 and Cys-188 were involved in mixed disulfides with glutathione. These data underline these two cysteines as the most reactive within each of the two Fc domains.

**DISCUSSION**

Antibodies constitute an excellent model system to investigate the folding of multimeric, multidomain proteins. These proteins contain both intra- and interdomain disulfide bonds, providing the possibility of elucidating the hierarchy of intra/interchain disulfide bond formation during the folding process. However, the analysis of the oxidative folding of the entire antibody molecule is hampered by several factors, including aggregation phenomena.

The analysis of immunoglobulin folding was then dissected by investigating the folding process of different portions of the antibody molecule. The elucidation of the folding pathway of the C-terminal immunoglobulin domain $\alpha 1$3 containing a single S-S bond had been the first step of a project aimed at analyzing the folding and oxidation of different parts of immunoglobulins. In the folding of $\alpha 1$3 the formation of the single disulfide occurs on the same time scale as the acquisition of the tertiary structure (18).

Here, the project was extended to the investigation of the folding pathway of the Fc fragment, consisting of the $\alpha 1$2 and $\alpha 3$3 domain of the heavy chain, each containing a single disulfide bond. The folding process was analyzed in vitro both in the absence and presence of PDI, i.e. under conditions that mimic...
those occurring in the endoplasmic reticulum. The extent of the reaction and the relative abundance of the folding intermediates were monitored by electrospray mass spectrometry.

The analysis of the un-catalyzed folding of the Fc fragment showed that the species containing one intramolecular disulfide (1S2H) predominated throughout the entire process, whereas the fully oxidized Fc fragment never accumulated in significant amounts. This result suggests the presence of a kinetic trap during Fc-folding that impairs the formation of the second S-S bond from the 1S2H species.

The assignment of disulfide bonds in the intermediates present at different times during the Fc-folding process revealed that the mixed disulfide involving Cys-188 and the exogenous glutathione formed at the early stages of the reaction and disappeared at late stages when the 1S2H component was the main species present in solution. On the other hand, Cys-24 present in the C_{i2} domain reacted much slower with the exogenous glutathione. Moreover, the 1S2H species was found to be a homogeneous intermediate only containing the Cys-130–Cys-188 disulfide belonging to the C_{i3} domain. Non-native, mixed disulfide is then attacked by Cys-130 to form the reaction of Cys-188 with the exogenous glutathione. This process at various steps, promoting the formation of the C_{i2} disulfide. The native species 2S containing both native disulfide bonds of the Fc molecule, started to accumulate very early and predominated at late stages of the process. Moreover, assignment of disulfide bonds at different times during the reaction revealed that both mixed disulfides, Cys-188-glutathione and Cys-24-glutathione, formed almost simultaneously at early stages. Finally, PDI does not change the folding pathway generating the same S-S bonds identified in the un-catalyzed process, as previously reported with different protein substrates (3).

This study demonstrates that the folding process of two adjacent immunoglobulin domains is remarkably different, although the tertiary structure is almost identical. It supports the view that the individual domains of antibodies have evolved to different end points concerning structure folding and stability while keeping the immunoglobulin fold intact.

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Hierarchical Formation of Disulfide Bonds in the Immunoglobulin Fc Fragment Is Assisted by Protein-disulfide Isomerase
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J. Biol. Chem. 2004, 279:15059-15066.
doi: 10.1074/jbc.M311480200 originally published online January 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311480200

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