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FULLY SYNTHETIC IMMUNOGENS. PART I. KINETIC STUDIES ON AIR OXIDATION OF THE HUMAN IgG1 BIS-CYSTEINYL FRAGMENT 225-232.

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Abstract: Air oxidation of the human IgG1 heavy chain bis-cysteinyl fragment 225-232 was found to produce in unexpected high yields (90%) the parallel dimer as it occurs in the native immunoglobulin molecule. Kinetics of air oxidation as well as of disproportionation of the antiparallel dimer via thiol disulfide interchange clearly revealed that thermodynamic control of folding, in terms of cysteine pairings, generates in remarkably preferred manner the native parallel structure albeit the small size of the protein fragment. The structural information inherent in the sequence suffices as driving force via short range interactions, to promote the parallel alignment. This local structure may thus play an important role as a chain folding initiation structure in the assembly pathway of immunoglobulins. These findings also simplify the synthetic accessibility of hinge-peptide 225-232/225'-232'-antigenic peptide conjugates as potential fully synthetic immunogens.

Introduction

In human IgG1 two vicinal interheavy chain disulfide bridges form a double-chain bis-cystinyl cyclic structure in parallel alignment whereby folding of this protein segment into a polyproline double helix[2] adds further rigidity to this unit within the flexible hinge region.

As discussed previously[3, 4] we have selected this compact cyclic structure as core molecule for the multiple attachment at the N- and/or C-termini of antigenic sequences in view of preparing fully synthetic immunogens possibly even in conformationally restricted form. This approach should allow to mimic surface epitopes of proteins in purposely designed miniproteins.

Synthetic studies on the hinge-peptide 225-232/225'-232'[5] revealed that the parallel alignment of the bis-cystinyl dimer cannot only be achieved via selective disulfide pairing procedures; but unexpectedly even air oxidation of the bis-cysteinyl-octapeptide was found to generate in surprisingly high yields the parallel dimer. This observation should greatly facilitate the synthesis of hinge-peptide/antigenic peptide conjugates in unambiguous parallel alignment since selective disulfide pairings, with the methods available today, require at least for one step iodine-mediated oxidation or sulfenyl halide based procedures. The iodine-method besides inducing possible disproportionation of existing disulfide bridges, leads to intermediate sulfenyl iodides which, depending upon steric restrictions, react with tryptophan residues if present in the antigenic peptide sequences, to produce

thio-indole derivatives[6]. Similar drawbacks are encountered with the sulfenyl halide methods[7, 8].

We have therefore investigated in detail the kinetics of oxidation of the bis-cysteinyl-octapeptide 225-232 as well as of disproportionation of the antiparallel to the parallel dimer in order to establish optimal conditions for dimerization in parallel alignment.

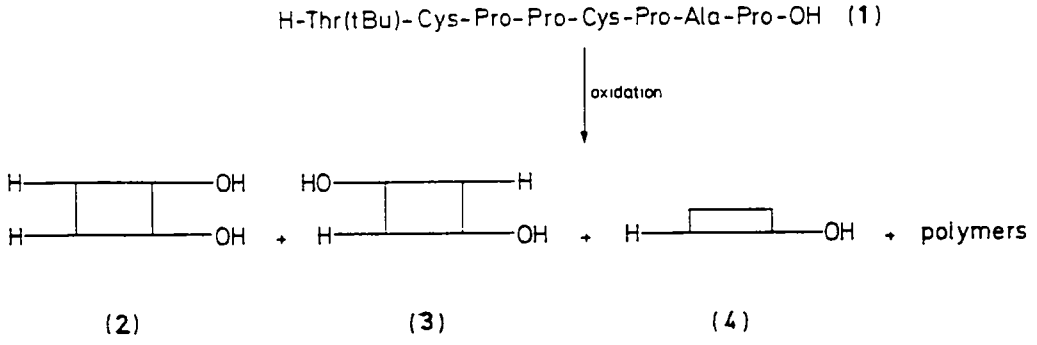
Results

Studies on air oxidation of the bis-cysteinyl peptides H-Cys-$(Gly)_n$-Cys-OH with n=0-15 have clearly shown that the nature of the oxidation products is mainly controlled by the probability of encounters of thiol groups when n≥4 [9-12]. Thus, under conditions of high dilution (10^{-3}-10^{-4}M) intrachain disulfide bridging with formation of ≥20- membered rings was predominant, as further confirmed by Sisido et al.[13]. Conversely, for n≤3 the peptide chain is not sufficiently flexible and thus, simple statistical theory cannot be applied: besides intrachain-bridged monomers, formation of dimers and polymers were observed[14]. Reduction of the ring size introduces stereochemical constraints and prediction of the product distribution becomes difficult, since secondary interactions among the peptide groups can induce energetically favoured structures. In fact stable 14-membered disulfide loops with n=2 are present as active sites in proteins involved in thiol-dependent redox reactions and in thiol-disulfide interchange processes, e.g. in thioredoxin with Gly-Pro[14, 15], in glutaredoxin with Pro-Tyr[16] and in protein disulfide isomerase with Gly-His[17] as intervening sequence between the two cysteine residues. The redox properties of these proteins have to be attributed to a considerable extent to the structural stability of these disulfide loops under different conditions. Nevertheless, synthetic studies on bis-cysteinyl-tetrapeptide derivatives related to thioredoxin and glutaredoxin[18-21] have shown that their oxidation under conditions of high dilution (10^{-3}M) with potassium ferricyanide generates, besides nonextractable polymers, a mixture of three oxidation products from which the cyclic monomer was isolated in yields of maximally 10-20%. Thus, mutual tertiary interactions are responsible for the exclusive formation of the disulfide loop in the folding process of these proteins.

In the heavy chain hinge-fragment 225-232 the intervening sequence between the two cysteine residues is Pro-Pro and the native disulfide structure of this portion of the IgG1 molecule is a parallel dimer. On the basis of above findings even in the present case, oxidation of the bis-cysteinyl-octapeptide **1** was expected to generate a mixture of compounds **2**, **3** and **4** with an additional amount of polymers (Scheme 1).

Oxidation of the bis-cysteinyl-octapeptide

From oxidation experiments with iodine[22, 23] as well as from the experiences gained in cyclization reactions in peptide chemistry it is known that the principle of high dilution is sufficiently preserved with concentrations in the range of 10^{-3}-10^{-4}M.



Scheme 1. Oxidation of the bis-cysteinyl-octapeptide corresponding to sequence 225-232 of human IgG1 and expected products.

Correspondingly, compound 1 was oxidized in dimethylformamide (3.10-3M) with equimolar amounts of azodicarboxylic acid di-tert-butyl ester. The oxidation reaction with diazene derivatives is known to proceed in two steps via intermediate formation of the sulfenohydrazide and subsequent displacement of the hydrazide by a second thiol group[24, 25]. The second step is particularly favoured in polar solvents. Thus, the polar aprotic anhydrous dimethylformamide was used as solvent and the cysteine peptide 1 was fully consumed within 60min; hplc analysis of the resulting reaction mixture revealed a product distribution with molar ratios for compounds 2:3:4 of 12:10:78. An approximately statistical distribution of the parallel and antiparallel dimer was obtained and as expected from the high dilution principle, a remarkable preference for the intrachain-bridged monomer. Since anhydrous conditions were selected, thiol-disulfide interchange as promoted by thiolate anions[26, 27] was widely suppressed. Therefore the resulting product distribution should reflect mainly a kinetically controlled oxidation. This is further confirmed by the results obtained in the oxidation experiment of the dithiol 1 with equimolar amounts of diazene in aqueous solution. Using again a concentration of peptide 1 of 3.10-3M its consumption was achieved within 10min and final 2:3:4 molar ratios of 40:20:40 were determined. The fast oxidation rate in water results from the presence of thiolate anions as highly reacting species in the diazene-mediated oxidation[27]. But the presence of this species is also known to provoke the thiol disulfide interchange which becomes a competing thermodynamically controlled side reaction. Correspondingly, partial interconversion of the oxidation products in disfavour of the cyclic monomer 4 occurs, leading to a preference of the parallel dimer 2 in respect to the antiparallel dimer 3.

By reducing the amount of diazene to a molar ratio of dithiol 1/oxidant of 1:0.5, the thiol disulfide interchange was purposely allowed to intervene for establishing conditions of

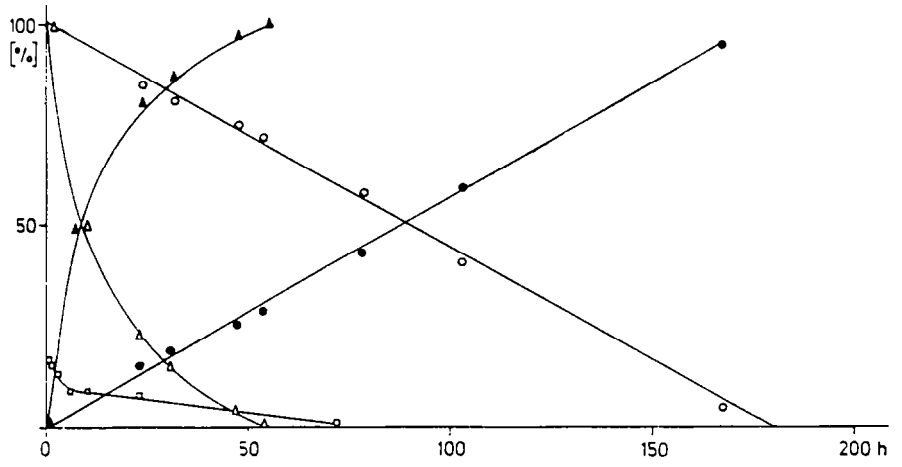


Figure 1. Oxidation of the bis-cysteinyl-peptide **1** by i) air oxygen in absence (O-O-O) and in presence of thioredoxin-S_2 at a molar ratio of **1**/thioredoxin of 10:1 (Δ-Δ-Δ); ii) azodicarboxylic acid dimorpholide at a molar ratio of **1**/oxidant of 1:0.5 (□-□-□); rates are expressed as percentage of residual dithiol **1**. Sum of the detected oxidation products formed in absence (●-●-●) and in presence of thioredoxin-S_2 (▲-▲-▲) as percentage of starting dithiol **1**.

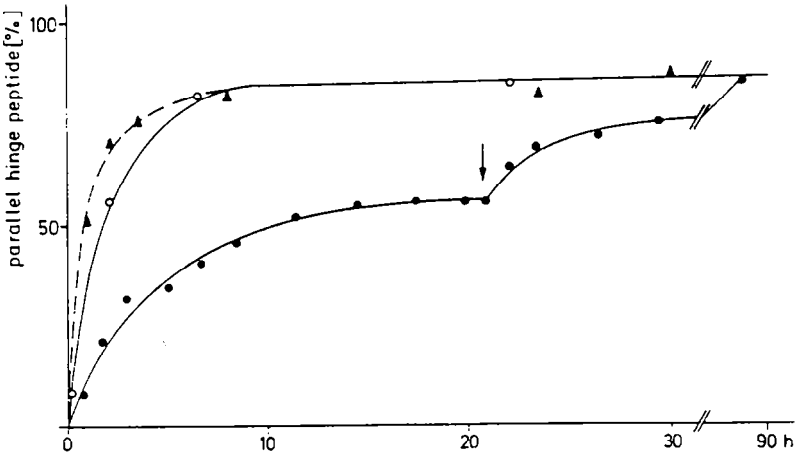


Figure 2. Thiol disulfide interchange of the antiparallel dimer **3** with i) the bis-cysteinyl-peptide **1** at a molar ratio of 5:1 in absence (O-O-O) and in presence of thioredoxin-S_2 at a molar ratio of **1**/thioredoxin of 1:0.1 (▲-▲-▲); ii) dithiothreitol at a ratio of **3**/DTT of 10:1 (●-●-●); the arrow indicates addition of a second portion of dithiothreitol again at the ratio of 10:1.

thermodynamic equilibrium and concomitant air oxidation served to achieve quantitative consumption of the dithiol **1**. The addition of subequimolar amounts of diazene was found to enhance remarkably the oxidation rate if compared with that obtained by air oxygen as discussed below and shown in Fig. 1. Nevertheless conditions of thermodynamic equilibrium were preserved and final **2**:**3**:**4** molar ratios of 90:8:2 were determined, i.e. a high preference for dimerization in parallel alignment as it occurs in the folding process of human IgG1. A similar but less efficient enhancement of the air oxidation rate was achieved by addition of dimethylsulfoxide as suggested by Fujii et al[28] (data not shown).

As mentioned above, oxidation of peptide **1** at a concentration of $3 \cdot 10^{-3}$M in aqueous solution (pH 6.8) by air oxygen under exclusion of metal ions was found to proceed at low rates (Fig. 1). Again simultaneous thiol disulfide interchange led at conditions of equilibrium to a product distribution of **2**:**3**:**4** of 90:8:2. Polymeric oxidation products were not detected by hplc and tlc; however these chromatograhic techniques may not be fully reliable for this purpose. On the other side the symmetry of the plots related to the consumption of the dithiol **1** and to the total amount of oxidation products (Fig. 1) indicates within the limits of error of the analytical techniques used, that polymeric structures were not produced at significant extents.

Addition of thioredoxin-S_2 at a molar ratio of peptide **1**/dithiol-disulfide oxidoreductase of 100:1 was without effect on the air oxidation rate; however a 10-fold increase of the thioredoxin-S_2 concentration was found to enhance remarkably the reaction rate (Fig. 1), but not to affect the product pattern.

Performing the air oxidation in alcaline media (pH 8.3) higher rates were observed as expected and formation of the cyclic monomer **4** was reduced to about half amount in favour of the parallel dimer **2**. Conversely, by lowering the concentration of the dithiol **1** to 10^{-4}M (pH 6.8) a 10-fold increase in the cyclic monomer **4** was induced and final **2**:**3**:**4** molar ratios of 70:10:20 were determined at conditions of equilibrium. Conversely air-oxidation of H-Cys-Gly-Gly-Cys-OH at a concentration of 10^{-4}M (pH 8.5) led to only 10% antiparallel dimer besides 15% monomer, the rest being oligomers[11].

Thiol-disulfide interchange

Since a thermodynamic control of folding, in the present case in terms of cysteine pairings, should lead to a product distribution at equilibrium which is insensitive to the pattern of the initial states, the antiparallel dimer **3** was exposed to thiol disulfide interchange at a concentration of $2.5 \cdot 10^{-3}$M (pH 6.8). For this purpose disproportionation of compound **3** via reduction and/or mixed disulfide formation, was induced by dithiothreitol at an arbitrary molar ratio of 10:1; in the time course of the reaction air oxidation was allowed to occur. The oxidative consumption of the thiol species by air oxygen was found to proceed at rates competitive with the thiol disulfide interchange reaction. Therefore additional amounts of dithiothreitol were necessary to reach conditions of equilibrium (Fig. 2). Quantification of the product pattern revealed molar ratios which were identical with

those detected in the air oxidation process, i.e. **2**:**3**:**4** = 90:8:2. This product distribution was also generated by incubating the antiparallel dimer **3** directly with the bis-cysteinyl-octapeptide **1** at a molar ratio of 5:1 to assure full equilibration even under conditions of simultaneous air oxidation. After 10h the dithiol **1** was consumed and conditions of equilibrium were reached (Fig. 2).

If the thiol disulfide interchange of the dimer **3** with the dithiol **1** was performed in presence of thioredoxin-S_2 at a dithiol **1**/biocatalyst 10:1 ratio an enhancement of equilibration rate was observed (Fig. 2), but no effect on the product distribution could be detected. Addition of dithiothreitol at the equilibration and oxidation endpoint to regenerate reduced thioredoxin did not further affect the molar ratios of the oxidized species.

Discussion

Air oxidation of the bis-cysteinyl-octapeptide 225-232 of the human IgG1 heavy chain under conditions of thermodynamic control was found to produce with surprising preference the dimeric structure in parallel alignment, whereas oxidation of the dithiol peptide under conditions of kinetic control led to a product distribution which reflects the high dilution principle and the statistical probability, i.e. a pronounced preference for the intrachain disulfide bridging and formation of the parallel and antiparallel dimers in a nearly 1:1 ratio. However, as long as thiol disulfide interchange was allowed for establishing the thermodynamic equilibrium, a product pattern of refolding in terms of cysteine pairings was obtained, which was independent of the starting products of the hinge-peptide system, thus indicating that the parallel dimer as present in the native IgG1 molecule, represents the energetically most favoured structure, even in absence of the stabilizing long range interactions of the folded protein.

It is known that thioredoxin as dithiol-disulfide oxidoreductase catalyzes efficiently the interchange of incorrect disulfides in scrambled proteins[29] in a manner similar to the protein disulfide-isomerase[30]. Addition of thioredoxin remarkably enhanced the rate of air oxidation of monomeric hinge-peptide, but a shift in the molar ratios of the oxidized species was not observed, thus suggesting the absence of strong kinetic barriers to reach conditions of equilibrium. Similarly, refolding of the scrambled hinge-peptide (antiparallel dimer) was also accelerated by thioredoxin.

Formation of the correct disulfide pattern in proteins occurs concomitantly with acquisition of the correct folded form and is driven by the thermodynamic stability of the native protein configuration. Nevertheless, thermodynamically stable local structures may play an important role in the initial stages of protein folding[31-34]. Thereby short range interactions are essentially implicated to promote stable core structures around which the rest of the protein chain will fold. These sequence-specific short range interactions may suffice for folding of isolated protein fragments as subdomains into stable native-like structures as well demonstrated recently with the bovine pancreatic trypsin inhibitor mono-cystinyl fragment 20-33/43-58 [35]. Similarly, sequence-specific information must be

the driving force for the observed predominant parallel alignment of the hinge-fragment 225-232/225'-232' in aqueous solution. Despite the small size of the protein fragment, this parallel dimer must correspond to an energetically highly favoured structure.

On the other side conformational studies on cystine containing cyclic tetrapeptides with the intervening sequences -Gly-Pro- and -Pro-Tyr- [21-23] and representing the redox-active disulfide loops of thioredoxin and glutaredoxin, respectively, led to the conclusion that highly folded β-turn type backbone conformations are responsible for the redox potential of these proteins. In their synthesis however, a predominant formation of the cyclic monomer was apparently not observed. Therefore we cannot exclude that in our hinge-peptide system the exocyclic portions of the molecule may contribute to the unique preference for parallel dimerization.

To conclude, above findings confirm that the hinge peptide 225-232/225'-232' with two vicinal interchain crosslinks represents a thermodynamically stable core molecule well suited for the design of synthetic miniproteins as potential immunogens. But the results also suggest that this local structure in the hinge corresponds to a pronounced free-energy minimum and thus possibly to an important "chain folding initiation structure" [36] in the assembly of immunoglobulins. This assumption is further supported by the observation that the initial step in covalent intermolecular crosslinking of immunoglobulins was disulfide formation between a nascent heavy chain and a completed chain, heavy or light depending on the cell type[37].

Experimental

Thioredoxin-S_2 was a gift of Dr. A. Holmgren, Karolinska Institute, Stockholm. Azodicarboxylic acid dimorpholide was prepared from the corresponding ethyl ester by known procedures[38]. Oxidation and thiol disulfide interchange reactions were carried out at room temperature and were monitored by hplc [μ-Bondapak C18; eluent: CH_3CN/0.1M sodium phosphate (pH 3.5); linear gradient from 13 to 40% CH_3CN in 50min; flow rate: 2.0ml/min; detection: UV at 210nm]. Integration of the peak areas of the thiol educt and of the oxidation products allowed for a quantitative determination of the product distribution.

The hplc retention times of H-Thr(tBu)-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH (**1**), of the parallel (**2**) and antiparallel (**3**) dimer [H-Thr(tBu)-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH]$_2$ as well as of the cyclic monomer (**4**) were determined with authentic samples. The syntheses of compounds **1**, **2** and **3** were reported previously[5].

Compound **4**, i.e. H-Thr(tBu)-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH was obtained as follows:

The octapeptide derivative **1**, i.e. H-Thr(tBu)-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH (86mg; 0.1mmol; peptide content: 98%) was reacted in argon-saturated DMF (33ml) with azodicarboxylic acid di-tert-butyl ester (23mg; 0.1mmol) at room temperature. As monitored by hplc under the chromatographic conditions reported above, the reaction was completed within 60min. Upon additional stirring for 1h, the solution was concentrated

and the product mixture was precipitated with ethyl acetate/diisopropyl ether and then chromatographed on a silica gel (230-400mesh) column (3 x 21cm) using $CHCl_3$/MeOH/AcOH/H_2O (60:25:2:4) as eluent. Fractions containing the desired product, as determined by tlc and hplc, were collected and concentrated, and the product was precipitated with ether. Yield: 30mg (30%; calcd. for a peptide content of 93%); homogeneous on tlc (solvent systems: $CHCl_3$/MeOH/AcOH/H_2O, 60:25:2:4; 1-propanol/AcOH/ pyridine/water, 60:6:24:20) and hplc (conditions as used for kinetic measurements); amino acid analysis (6M HCl containing 2.5% thioglycolic acid at 110°C for 24h): Thr 0.85(1) Pro 3.98(4) Ala 1.00(1) Cys 2.14(2); peptide content: 93% calcd. for M_r=839.1; the low recovery of Thr upon acid hydrolysis has been previously discussed[5]. The monomeric structure was determined by the method of least substitution via reaction with 2,4-dinitrofluorobenzene[39].

Oxidation of compound **1**, i.e. of H-Thr(tBu)-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH was performed as follows:

a) a solution of the bis-cysteinyl-octapeptide **1** (1.03mg; 1.2μmol; peptide content: 98%) in 0.4ml 0.1M ammonium acetate (pH 6.8) was gently stirred at room temperature under air in absence and in presence of thioredoxin-S_2 (0.12μmol or 0.012μmol). Kinetics of oxidation of **1** (Fig. 1) and the molar ratios of the resulting oxidation products were determined by hplc of aliquots of the reaction mixture.

b) the bis-cysteinyl-octapeptide **1** (3.8mg; 4.4μmol; peptide content: 98%) was reacted in 1.5ml H_2O with azodicarboxylic acid dimorpholide at molar ratios of 1:1 and 1:0.5. The reaction mixtures were kept under argon in the first and under air in the second experiment. The oxidation reaction was followed by hplc as described for a). The results are reported in Fig. 1.

The thiol disulfide interchange of the antiparallel dimer [H-Thr(tBu)-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH]$_2$ was carried out as follows:

a) an argon-saturated solution of the antiparallel dimer **3** (2.2mg; 1.2μmol; peptide content: 89.5%) and of the bis-cysteinyl-octapeptide **1** (0.2mg; 0.24μmol; peptide content: 98%) in 0.48ml 0.1M ammonium acetate (pH 7.0) was gently stirred in absence and in presence of thioredoxin-S_2 (0.12μmol). In the time course of the reaction air oxidation was allowed for consumption of the dithiol **1**. Conversion of the antiparallel dimer **3** to the parallel dimer **2** was followed by hplc as described above (Fig. 2).

b) the antiparallel dimer **3** ($2.5 \cdot 10^{-3}$M in 0.1M ammonium acetate, pH 7.0) was reacted with dithiothreitol at a molar ratio of 10:1. As for a) air oxidation was allowed for consumption of the thiol species. Upon reaching plateau levels for the parallel dimer formation, a second portion of dithiothreitol was added. Kinetics of the thiol disulfide interchange are reported in Fig. 2.

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