Re-formation of disulphide bonds in reduced antithrombin III

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Human antithrombin III (AT-III) contains three disulphide linkages (Cys-8–Cys-128, Cys-21–Cys-95 and Cys-247–Cys-430), and two of them (Cys-8–Cys-128 and Cys-21–Cys-95) are situated near the heparin-binding domain of the inhibitor. We demonstrate in this paper that: (i) partially reduced AT-III (with Cys-8–Cys-128 and Cys-21–Cys-95 quantitatively reduced) could be re-oxidized in air to regain 70–80% of its heparin cofactor activity and thrombin-inhibitory activity; (ii) completely reduced AT-III was re-oxidized under similar conditions and recovered 30–35% of its biological activities. Structural analysis of refolded AT-IIIs indicates that restorations of their disulphide contents and conformations (evaluated by chemical modification) are congruent with recoveries of their biological activities.

INTRODUCTION

Antithrombin III (AT-III) is a glycoprotein found in mammalian plasma that inhibits thrombin and other serine proteinases comprising the coagulation cascade (for review see Travis & Salvesen, 1983). Inhibition of thrombin by this inhibitor is greatly accelerated in the presence of heparin, a sulphated polysaccharide (Rosenberg, 1985). Although the molecular mechanism of heparin function remains to be clarified in detail, it has been clearly shown that heparin binding to AT-III is a prerequisite for its enhancing effect (Einarsson & Andersson, 1977; Villanueva & Danishefsky, 1977; Nordenman & Björk, 1978; Olson *et al.*, 1981) and that this binding induces a conformational change of AT-III that leads to the exposure of Lys-236 for chemical modification (Chang, 1989). A considerable amount of work has also been directed at the elucidation of heparin binding site in AT-III, and it has shown that Pro-41 (Chang & Tran, 1986), Arg-47 (Koide *et al.*, 1984) and Trp-49 (Blackburn *et al.*, 1984) of the inhibitor are essential for heparin binding and that Lys-107, Lys-114, Lys-125 and Lys-136 are involved in direct interaction with heparin (Peterson *et al.*, 1987; Liu & Chang, 1987; Chang, 1989).

AT-III is a single-chain polypeptide with a molecular mass of 58000 Da (Nordenman *et al.*, 1977). There are six cysteine residues, which form three disulphide linkages (Petersen *et al.*, 1979). Under mild reduction conditions one of the three disulphide bonds could be selectively cleaved, resulting in a decreased affinity for heparin and the loss of heparin-accelerated thrombin-inhibitory activity (Longas *et al.*, 1980). More recently we have shown that the three disulphide linkages of AT-III could be sequentially reduced, with Cys-8–Cys-128 being the most sensitive, followed by Cys-21–Cys-95, with Cys-247–Cys-430 being the most resistant to reduction (Sun & Chang, 1989a). The reduction of Cys-8–Cys-128 was found to correlate quantitatively with the loss of the heparin cofactor activity of AT-III. These findings confirm the crucial role of disulphide linkages in AT-III and manifest the feasibility of preparing chemically modified AT-III that has a well-defined content of disulphide bonds. In order to investigate further the structure–function relationship of AT-III, we demonstrate in the present paper that partially and completely reduced AT-III can be re-oxidized in air with correct disulphide pairing to restore its biological activity.

EXPERIMENTAL

Materials

AT-III was purified from human plasma by using heparin–Sephadex chromatography (Koide, 1979). Human α -thrombin (catalogue no. T-6795; > 3000 NIH units/mg) and low-molecular-mass heparin (catalogue no. H-5640; 4000–6000 Da) were purchased from Sigma Chemical Co. S-DABIA was prepared by the method described previously (Sun & Chang, 1989a). S-DABITC was synthesized by the method of Chang (1989). Dithioerythritol and tributylphosphine were from Merck, 2-mercaptoethanol was from Serva and CNBr was from Fluka. Chromozym TH was obtained from Boehringer Mannheim.

Preparation of time-course-re-oxidized AT-III

AT-III (1.25 mg) was incubated with various concentrations of dithioerythritol (1 mM, 10 mM and 100 mM) in 1.25 ml of 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.2, at room temperature and under N₂ for 45 min. A 20% portion (250 μ g) of the sample was withdrawn from the mixture and carboxymethylated with iodoacetic acid to block the free thiol groups, and this sample was designated the zero-time control. Re-oxidation started by passing the remainder of the sample (1 mg) through a PD-10 column pre-equilibrated in 50 mM-sodium bicarbonate buffer, pH 9.0. Elution was completed within 2 min. The eluate (in 2.4 ml) was mixed immediately with 10 μ l of 0.1% (v/v) 2-mercaptoethanol (Epstein & Goldberger, 1963) and left at room temperature open to the air. At time intervals (10 min, 20 min, 30 min and 60 min) portions (600 μ l) of the sample were removed and re-oxidation was terminated by adding iodoacetic acid. Carboxymethylated samples were desalted on PD-10 columns pre-equilibrated in 50 mM-ammonium bicarbonate buffer, pH 8.0, and freeze-dried. Altogether there were 15 re-oxidized samples. An additional control treatment of AT-III was performed in parallel without the addition of dithioerythritol. The structures and biological activities of the re-oxidized samples were analysed by the following methods.

Characterization of the disulphide content of re-oxidized AT-III

This was performed by using a thiol-specific colour reagent, S-DABIA, with the strategy described previously (Sun & Chang,

Abbreviations used: AT-III, human antithrombin III; S-DABIA, 4-*NN*-dimethylamino-4'-iodoacetamido-azobenzene-2'-sulphonic acid; S-DABITC, 4-*NN*-dimethylamino-4'-isothiocyanato-azobenzene-2'-sulphonic acid.

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1989a). AT-III (5 nmol) was dissolved in 50 μ l of 0.25 M-sodium bicarbonate buffer, pH 8.2, and 25 μ l of propan-1-ol. The solution was flushed with N₂, and to it was added 300 nmol of tributylphosphine (in 10 μ l of propan-1-ol). After 3 h at room temperature the sample was mixed with S-DABIA (1500 nmol in 300 μ l of 50 mM-sodium bicarbonate buffer, pH 9.0), and the mixture was incubated for a further 1 h at room temperature in the dark. Excess reagent was removed by desalting on a PD-10 column, and coloured protein was collected and freeze-dried. S-DABIA-modified AT-III was then digested with trypsin (enzyme/substrate weight ratio 1:20) in 100 μ l of 50 mM-ammonium bicarbonate buffer, pH 8.0, overnight, and was directly injected for h.p.l.c. analysis (Fig. 2). The recoveries of coloured cysteine-containing peptide were taken to be a measure of the extent of re-oxidation (Figs. 2 and 3).

S-DABITC modification of re-oxidized AT-III (Chang, 1989)

AT-III (5 nmol) was dissolved in 0.25 ml of 50 mM-sodium bicarbonate buffer, pH 8.3, and the sample was then added to an equal volume of S-DABITC solution (2 mM in 50 mM-sodium bicarbonate buffer). Formation of the derivative was allowed to proceed at 22 °C for 7.5 min. The reaction was terminated by passing the sample through a PD-10 column pre-equilibrated in 50 mM-ammonium bicarbonate buffer, pH 8.0, and freeze-dried. The modified AT-III was reduced with dithioerythritol in 0.5 M-Tris/HCl buffer, pH 8.4, containing 5 M-guanidinium chloride and then treated with iodoacetic acid. Carboxymethylated samples were again desalted on PD-10 columns, freeze-dried and digested overnight with trypsin (enzyme/substrate weight ratio 1:20) in 250 μ l of 50 mM-ammonium bicarbonate buffer, pH 8.0. The digested samples were directly loaded for h.p.l.c. analysis (Fig. 4).

Preparation of C-1 fragment for CNBr cleavage of AT-III (Rosenfeld & Danishefsky, 1986)

AT-III (2 mg) was dissolved in 0.5 ml of 70% (v/v) formic acid and mixed with 8 mg of CNBr. The solution was flushed with N₂ for 1 min, and left at room temperature for 24 h in the dark. Water (12 ml) was added and the diluted sample was freeze-dried. The sample was dissolved in 2 ml of 0.1 M-formic acid and applied on to a Sephadex G-50 column (2 cm $\times$ 90 cm) pre-equilibrated with 0.1 M-formic acid. The first fraction (C-1) contains three polypeptides (residues 1–17, 104–251 and 424–432) linked by Cys-8–Cys-128 and Cys-247–Cys-430. This fraction was collected and characterized by amino acid analysis and quantitative N-terminal analysis.

Structural analysis of polypeptides

Quantitative N-terminal analysis of polypeptides was performed by the dimethylaminoazobenzene isothiocyanate method (Chang, 1988). Amino acid sequence was determined with a gas-phase sequencer (Hunkapiller *et al.*, 1983).

Measurement of antithrombin activity

This was performed basically as described by Björk & Nordling (1979). Antithrombin activity was determined as the ability to inhibit thrombin-catalysed hydrolysis of Chromozym TH in the absence and in the presence of low-molecular-mass heparin. For heparin cofactor activity, 4 pmol of thrombin, 6 pmol of AT-III and 200 pmol of heparin were incubated in 50 μ l of 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.2, at room temperature for 2 min. For progressive inhibitory activity, 4 pmol of thrombin and 7.5 pmol of AT-III were incubated in the same buffer for 15 min. The residual thrombin activity was determined by transferring 25 μ l of the incubated mixture to a photometric cuvette that already contained 1 μ mol of Chromozym TH in

975 μ l of the aforementioned NaCl/Tris/HCl buffer. The rate of the release of *p*-nitroaniline from the substrate was monitored at 405 nm for 2 min. The specific batch of low-molecular-mass heparin (Sigma Chemical Co. catalogue no. H-5640) used in this study had heparin cofactor activity comparable with that of unfractionated heparin (Sigma Chemical Co. catalogue no. H-3250) isolated from the same source (pig intestinal mucosa) (Sun & Chang, 1989b). We have chosen the low-molecular-mass heparin because in analyses of the structure of the heparin-binding site of AT-III low-molecular-mass heparin is less likely to produce non-specific protection.

RESULTS

Thrombin-inhibitory activities of re-oxidized AT-III

The heparin cofactor activity and the progressive inhibitory activities of time-course-re-oxidized AT-III are shown in Fig. 1. There was a parallel recovery of both activities during the course of re-oxidation. Samples reduced in 1 mM- or 10 mM-dithioerythritol lost 60–85% of their thrombin-inhibitory activity both in the absence and in the presence of heparin (Fig. 1, zero-time samples). They regained up to 70–86% of the activities after 60 min re-oxidation. The sample reduced in 100 mM-dithioerythritol, however, recovered only about 43% of its thrombin-inhibitory activity and 27% of its heparin cofactor activity after the same period of re-oxidation. Prolonged re-oxidation beyond 60 min allowed further but very slow recovery of the activities. We have confined our experiment within 1 h in order to compare the re-oxidation rates of AT-III that had undergone different degrees of reduction (in 1 mM-, 10 mM- and 100 mM-dithioerythritol).

Analysis of disulphide bonds of re-oxidized AT-III

Disulphide bonds of re-oxidized AT-III were quantified by the method of Sun & Chang (1989a). Free thiol groups of a partially re-oxidized AT-III were blocked by iodoacetic acid, and the disulphide bonds were then completely reduced and labelled with S-DABIA. A colour peptide mapping subsequently revealed the content of cysteine residues that were engaged in the disulphide linkages. Examples of original results are illustrated in Fig. 2. A control AT-III contains six cysteine residues, which form three

Fig. 1. Biological activities of re-oxidized AT-III

The heparin cofactor activity (—) and the thrombin-inhibitory activity (-----) of re-oxidized AT-III were measured by the methods indicated in the Experimental section. AT-III was first reduced in 1 mM- (●), 10 mM- (▲) and 100 mM-dithioerythritol (■) for 45 min. Refolding was started by desalting on a PD-10 column and terminated by carboxymethylation with iodoacetic acid. Both activities obtained from the control AT-III were taken as 100%.

Fig. 2. Colour cysteine-containing-peptide mapping of re-oxidized AT-III that had been reduced in 1 mM-dithioerythritol

Control AT-III and re-oxidized AT-III (after blocking of thiol groups by iodoacetic acid) were completely reduced and modified by reaction with S-DABIA. The samples were digested with trypsin (enzyme/substrate weight ratio 1:20) in 50 mM-ammonium bicarbonate buffer, pH 8.0, at room temperature overnight, and 10 μ g of the digested AT-III was subjected to h.p.l.c. (a) Control AT-III; (b) zero-time-re-oxidized sample; (c) 10 min re-oxidation; (d) 20 min re-oxidation; (e) 30 min re-oxidation; (f) 60 min re-oxidation. Solvent A was 10 mM-ammonium acetate buffer, pH 5.0, and solvent B was acetonitrile. The gradient was 15% (v/v) solvent B isocratically for 5 min, 15% (v/v) solvent B to 40% (v/v) solvent B from 5 to 45 min, 40% (v/v) solvent B to 80% (v/v) solvent B from 45 to 50 min, held at 80% (v/v) solvent B for 5 min, then returned to 15% (v/v) solvent B in 2 min. The column was Vydac C_{18} (particle size 5 μ m) for peptides and proteins. The column temperature was 23 $^{\circ}$ C. Cysteine-containing peptides were monitored at 436 nm. Sequences of cysteine-containing peptides were characterized previously (Sun & Chang, 1989). Those peptides that were derived from non-specific cleavage (*Cys-21 and *Cys-95) are marked by asterisks. Disulphide pairings are indicated above panels (a) and (d).

disulphide bonds (Fig. 2a). Cys-21 and Cys-95 gave double peaks because of non-specific cleavages (Sun & Chang, 1989a). Partial reduction in 1 mM-dithioerythritol resulted in cleavages of about 95% of Cys-8-Cys-128, 80% of Cys-21-Cys-95 and 20% of Cys-247-Cys-430 (Fig. 2b, zero-time re-oxidized sample). Throughout the course of re-oxidation (10 min, 20 min, 30 min and 60 min; Figs. 2c-2f), recoveries of all cysteine-containing peptides increased, indicating the re-formation of disulphide bonds. For quantitative analysis, yields of cysteine-containing peptides in Fig. 2(a) were taken as 100%. The results are summarized in Fig. 3. By increasing the concentration of dithioerythritol from 1 mM to 100 mM, the three disulphide linkages of AT-III could be sequentially reduced (see zero-time samples in Figs. 3a, 3b and 3c). During the process of re-oxidation, the Cys-8-Cys-128 and Cys-21-Cys-95 disulphide bonds re-formed at comparable rates and reached 70-80% after 60 min. With the samples reduced in 1 mM- and 10 mM-dithioerythritol, the extent of disulphide bond re-formation agrees well with the recoveries of their biological activities (Fig. 1). It is relevant to mention that the results of peptide analysis alone do not confirm correct pairing of disulphide bonds, except for the sample reduced in 1 mM-dithioerythritol (Fig. 3a), in which the extent of reduction of the three disulphide bonds could be clearly distinguished.

We have also investigated the effect of heparin (weight ratio to AT-III 2:1) on the re-oxidation process of AT-III that had been reduced in 1 mM-dithioerythritol, and found that heparin did not accelerate the rate of disulphide bond re-formation (X.-J. Sun & J.-Y. Chang, unpublished work).

Monitoring the conformation of re-oxidized AT-III

The conformation of AT-III can be monitored by using the method of chemical modification. In native AT-III two lysine residues located within the heparin-binding site (Lys-125 and Lys-136) are preferentially modified by S-DABITC, a lysine-specific colour reagent (Chang, 1989) (Fig. 4a). Upon binding of heparin, Lys-125 and Lys-136 are shielded from modification, and at the same time Lys-236 is exposed for S-DABITC labelling as a consequence of the conformational change of AT-III (Fig. 4b). Regardless of the source of heparin, the shielding of Lys-125 and Lys-136 has been found to be inversely proportional to the unmasking of Lys-236 (Chang, 1989). Furthermore, complete reduction and carboxymethylation renders Lys-136 and Lys-236 totally inactive towards modification by S-DABITC (X.-J. Sun & J.-Y. Chang, unpublished work). The reactivities of Lys-136 and Lys-236 thus serve as useful indicators for both the affinity of heparin binding and the conformational change induced by bound heparin. We have found that with re-oxidized AT-III the reactivities of these two lysine residues are closely related to their regained biological activities (Fig. 1) and disulphide contents (Fig. 3). Taking AT-III reduced in 1 mM-dithioerythritol as an example, after 60 min re-oxidation Lys-236 exhibited 67% of the reactivity (as compared with the control sample) in the absence of heparin (Fig. 4c), and it recovered 75% of the reactivity in the presence of heparin (Fig. 4d). The same re-oxidized sample regained approx. 65-80% of its heparin cofactor activity, thrombin-inhibitory activity (Fig. 1) and disulphide content (Fig. 3a).

Fig. 3. Quantitative determination of six cysteine residues that are engaged in disulphide linkages during the process of re-oxidation of reduced AT-III

AT-III was reduced in 1 mM-dithioerythritol (a), 10 mM-dithioerythritol (b) and 100 mM-dithioerythritol (c). The results were obtained from the analysis of chromatograms as illustrated in Fig. 2: ○, Cys-8; ▲, Cys-21; △, Cys-95; ●, Cys-128; ■, Cys-247; □, Cys-430. The recoveries of cysteine-containing peptides from the control sample were taken as 100%.

Quantitative *N*-terminal analysis of C-1 fragment derived from CNBr cleavage of AT-III

The largest CNBr-cleavage fragment of AT-III contains three polypeptides that linked together by two disulphide linkages. Peptide 1 (residues 1–17, *N*-terminus histidine) and peptide 3 (residues 424–432, *N*-terminus glycine) are connected to peptide 2 (residues 104–251, *N*-terminus glutamic acid) through Cys-8–Cys-128 and Cys-247–Cys-430 disulphide bonds respectively. With native AT-III these three peptides are expected to be present in equal molar proportions in fragment C-1, and they can be evaluated by quantitative *N*-terminal analysis. With re-oxidized AT-III the recoveries of these three peptides are dependent upon the re-formation of disulphide bonds, and the molar ratios of peptide 1/peptide 2 (*N*-termini histidine/glutamic acid) and peptide 3/peptide 2 (*N*-termini glycine/glutamic acid) can be used to monitor the extent of pairing of Cys-8–Cys-128 and Cys-247–Cys-430 respectively. The results are shown in Fig. 5. For native AT-III the molar ratios of histidine/glutamic acid

Fig. 4. Monitoring of the conformation of AT-III by the method of chemical modification

(a) and (b) Control AT-III; (c) and (d) AT-III reduced in 1 mM-dithioerythritol followed by 60 min re-oxidation; (e) and (f) AT-III reduced in 100 mM-dithioerythritol followed by 60 min re-oxidation. Samples were modified with 1 mM-S-DABITC in the absence of heparin (a, c and e) or in the presence of heparin (b, d and f) at room temperature for 7.5 min, denatured and digested with trypsin. Peptides containing S-DABITC-labelled lysine residues were detected at 436 nm. In the absence of heparin two major coloured peptides T1 (residues 133–139) and T2 (residues 115–129), corresponding to labelling at Lys-136 and Lys-125 respectively, were observed (Chang, 1989). In the presence of heparin Lys-125 and Lys-136 were protected from modification, but a conformational change of AT-III induced by heparin binding exposed Lys-236 for S-DABITC modification (peptide Ta, residues 236–241). Solvent A was 17.5 mM-acetate buffer, pH 5.0, and solvent B was acetonitrile. A linear gradient of 10% (v/v) solvent B to 70% (v/v) solvent B in 30 min was applied. The column was Vydac C₁₈ for peptides and proteins. The column temperature was 22 °C. The flow rate was 1 ml/min.

and glycine/glutamic acid *N*-termini are 1.07:1 and 1.82:1 (the recovery of glycine was higher than expected, and we suspect that this is a result of contamination). These values are decreased with the C-1 fragment of re-oxidized AT-III. Again, we observe that the decreased molar ratio of histidine/glutamic acid *N*-termini, which reflects the mis-pairing of Cys-8–Cys-128, is in congruence with the diminished biological activity (Fig. 1), decreased disulphide content (Fig. 3) and impaired reactivity of Lys-136 and Lys-236 (Fig. 4).

Fig. 5. Quantitative *N*-terminal analysis of CNBr-cleavage fragment C-1 derived from control AT-III and two re-oxidized AT-IIIs (reduced in 1 mM-dithioerythritol and 100 mM-dithioerythritol followed by 60 min re-oxidation)

The samples were cleaved by CNBr as described in the Experimental section. After separation of CNBr-cleavage fragments on a Sephadex G-50 column, fraction C-1 was collected and *N*-termini were determined by the DABITC method (Chang, 1988). Data are presented by the molar ratios of histidine/glutamic acid and glycine/glutamic acid *N*-termini.

DISCUSSION

The reversible reduction and re-oxidation of disulphide bonds of biologically active proteins have been used to study the folding pathway of several proteins, and particularly detailed information has been obtained from the case of bovine pancreatic trypsin inhibitor (for review see Creighton, 1986). In the present paper we demonstrate that both partially or completely reduced AT-III could be re-oxidized to regain its biological activity. The criteria for judging the refolding of AT-III were based on: (1) the recovery of its biological activities, both heparin cofactor activity and thrombin-inhibitory activity; (2) the re-formation of correct disulphide bonds; (3) the restoration of the heparin-binding site as judged by the technique of chemical modification.

Human AT-III contains three disulphide bonds. Two of them (Cys-8–Cys-128 and Cys-21–Cys-95) are located within the *N*-terminal domain, which had been shown to comprise the heparin-binding site (Blackburn *et al.*, 1984; Koide *et al.*, 1984; Chang & Tran, 1986; Peterson *et al.*, 1987; Liu & Chang, 1987; Chang, 1989). The other (Cys-247–Cys-430) is situated within the C-terminal domain, which forms a large disulphide loop that contains the reactive site of the inhibitor (Björk *et al.*, 1981). Partial reduction of AT-III (in 1 mM- and 10 mM-dithioerythritol) disrupted mainly the two disulphide bonds located within the *N*-terminal domain. These partially reduced AT-IIIs were re-oxidized to recover about 70% of the biological activity, disulphide content and native conformation as judged by chemical modification. Completely reduced AT-III, however, recovered only about 30% of its biological activity (Fig. 1) and 35% of the reactivities of Lys-136 and Lys-236 towards S-DABITC (see Figs. 4e and 4f), although greater than 50% of its disulphide bonds were re-formed (Fig. 3c). These data, together with results obtained from the analysis of CNBr-cleavage fragment C-1 (Fig. 5), suggest the occurrence of random disulphide pairing, generating 'scrambled AT-III' devoid of native conformation and biological activity. It is also likely that correct disulphide pairing alone is insufficient to ensure restoration of the native con-

formation on re-oxidation of reduced AT-III. Certain essential structures that were unfolded as a consequence of complete reduction may not be able to refold properly under the re-oxidation conditions described. Villanueva & Allen (1983a,b) investigated the refolding properties of AT-III unfolded in guanidinium chloride. Two unfolding domains in AT-III during its denaturation have been discovered. The first unfolding domain of low stability (midpoint at 0.7 M-guanidinium chloride), which is believed to be important to the heparin cofactor activity, is irreversible upon renaturation, and the second unfolding domain (midpoint at 2.3 M-guanidinium chloride) is reversible. Their results also suggest that structural elements in addition to the correct disulphide linkages are required to maintain the proper conformation of AT-III.

The location of the heparin-binding site in AT-III has come to light in the last few years (Rosenfeld & Danishefsky, 1986; Smith & Knauer, 1987). In particular, a lysine cluster (Lys-107, Lys-114, Lys-125 and Lys-136) has been implicated as participating in direct heparin binding (Peterson *et al.*, 1987; Liu & Chang, 1987; Chang, 1989). More recently we have shown that the disulphide bridge (Cys-8–Cys-128) that is attached to this lysine cluster is the most sensitive towards reduction, and its intactness is required for the heparin cofactor activity of AT-III (Sun & Chang, 1989a). In the present study we clearly demonstrate that breakage of Cys-8–Cys-128 as well as Cys-21–Cys-95 can be reversed by re-oxidation in air, and the biological activity can be recovered. This finding further confirms our early suggestion that Cys-8–Cys-128 is essential for the integrity of the heparin-binding sites of AT-III.

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