REGENERATION OF NATIVE STRUCTURE AND BIOLOGICAL ACTIVITY BY AIR OXIDATION OF REDUCED PANCREATIC SECRETORY TRYPSIN INHIBITOR AND Des–Thr–Ser–Pro–Gln–Arg-INHIBITOR

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1. Introduction

Pancreatic secretory trypsin inhibitors (KAZAL-typ [1]) selectively inhibit the proteolytic enzyme trypsin by formation of stoichiometric 1:1 molar complexes [2–5]. These inhibitors are low-molecular weight proteins [3, 6], which are particular good examples of proteins with distinct biological function [4]. The amino acid sequences of the bovine [7, 8], porcine [9, 10] and ovine [11] inhibitors have been determined. As could be demonstrated by ultraviolet spectroscopy [6] and denaturation and reduction experiments [7–9], a discrete three dimensional structure is a prerequisite for inhibitory activity. The native structure is maintained by three disulfide bonds. Inactivation of the trypsin-inhibiting activity occurs upon denaturation and reduction of the disulfide bonds. This paper provides evidence that the native inhibitor exhibits a definite α-helix content, which almost disappears upon denaturation in urea and upon reduction with dithiothreitol. The total activity may rapidly be restored by air reoxidation of the sulfhydryl groups regenerating the native structure as measured by optical rotatory dispersion. The results of comparative studies on porcine inhibitors, I, II and Des–Thr–Ser–Pro–Gln–Arg-inhibitor [12] demonstrate that the last five amino acids of the amino terminal sequence of fully reduced inhibitor I are not necessary to induce correct folding and to restore the inhibitory activity upon reoxidation.

2. Materials and methods

The pancreatic secretory trypsin inhibitors I and II and Des–Thr–Ser–Pro–Gln–Arg-inhibitor were isolated from porcine pancreatic glands [6–12]. The activity of the inhibitor was measured by its reaction with trypsin. The trypsin activity was determined by photometric assay at 405 nm of the rate of hydrolysis of α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide from Fluka AG, Buchs, Switzerland, at pH 7.8 in 0.2 M triethanolamine buffer (0.01 M $CaCl_2$) at 25° [5].

The trypsin inhibitors were denatured in 8 M urea or guanidine and reduced with dithiothreitol under a barrier of nitrogen at 20° according to the method of Crestfield [13]. Inhibitor (3 mg) was dissolved in 1 ml of 1 M tris-HCl buffer pH 8.6. Recrystallized and deionized urea (1.2 g) and 0.4 ml of a solution of 5% EDTA in water were added and the mixture reacted with 38 mg of dithiothreitol for 4 hr. The mixture was acidified to pH 3.5 with acetic acid, and the reduced proteins were freed of the reagents by desalting on a column of Bio Gel P-2 (0.9 × 100 cm) previously equilibrated with 0.1 M acetic acid. The protein fraction was lyophilized. The material isolated was tested for inhibitory activity. An average of 2% of the initial specific activity was found in the test, which was either residual or regenerated activity.

The reoxidation of the sulfhydryl groups was achieved at 20° by bubbling air through a solution of denatured and reduced inhibitor (2.8 mg) in 5.6 ml 0.02 M phosphate buffer pH 8.0. Aliquots of 0.2 ml were withdrawn at zero time after dissolution and at intervals, diluted to 10 ml with water and tested for inhibitory activity.

Optical rotatory dispersion measurements were performed with a recording spectrophotometer, Cary model 60 (Applied Physics Corp., Monrovia, U.S.A.). Fused thermostated cells (Perkin Elmer, Überlingen, Germany) of path length 1 cm were used. Mean residue rotations were calculated using the formula [14]:

$$[R'] = \frac{3}{n_\lambda^2 + 2} \times \frac{M_R}{100} [\alpha]_\lambda$$

where n_λ is the refractive index of the solvent at wavelength λ and M_R the mean molecular weight of an amino acid residue in the inhibitor protein (110) and $[\alpha]_\lambda$ the specific optical rotation at wavelength λ, $[\alpha] = \alpha/cd$.

The protein concentrations were determined by optical absorbance at 276 nm using the molar extinction coefficient for native inhibitor I $\epsilon^{1\,cm}_{276\,nm} = 3300$ M^{-1} cm^{-1} at pH 7.8. The concentration of the denatured and reduced inhibitor I was determined by dry weight.

The optical rotatory dispersions were measured in 0.1 M phosphate buffer pH 6.0 at 20°. A refractive index of $n_{233\,nm} = 1.3878$ was used for calculations of the mean residue rotations of native and reoxidized inhibitor I. The measurements on denatured and reduced inhibitor were performed in the same buffer, but in the presence of 8 M guanidine and dithiothreitol. For calculations a refractive index $n^{guan.}_{233\,nm} = 1.4919$ was used.

The α-helix content was calculated from the depth of the trough of the rotatory dispersion using the relation of Simmons et al. [15]:

$$f = \frac{[R']^{prot.}_{min} - [R']^{coil}_{min}}{[R']^{helix}_{min} - [R']^{coil}_{min}}$$

The mean residue rotation used for random coil was $[R']^{coil}_{min} = -1750$ [15–17]. For 75% helical structure a value of $[R']^{75\%\,helix}_{min} = -9000$ was used based on calculations of the helix content of $f_{Mb} = 75\%$ for sperm whale myoglobin [15].

3. Results and discussion

The porcine pancreatic secretory trypsin inhibitors I (56 residues) and II (52 residues) and Des–Thr–Ser–Pro–Gln–Arg-inhibitor (inhibitor fragment) were inactivated upon denaturation in 8 M urea or guanidine. Subsequent removal of the denaturing agents without prior reduction of the disulfide bonds completely restored the trypsin inhibiting activity [6]. The fully reduced inhibitors retained less than 2% activity after removal of the denaturing and reducing agents. The activity of all three inhibitors could, however, be regained quickly after reduction by air reoxidation. The specific activity of the reduced inhibitors I and II was restored completely (100%) after a minimum period of 80 min, that of reduced Des–Thr–Ser–Pro–Gln–Arg-inhibitor after only 40 min (fig. 1).

As was indicated by the ultraviolet Cotton effect, a definite three dimensional structure is allied with the biological activity. The curves of the optical rotatory dispersion of native inhibitor I, of the reduced protein

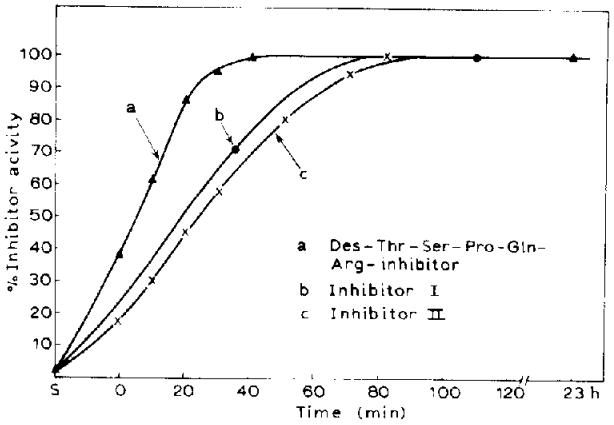

Fig. 1. Reoxidation of reduced pancreatic secretory trypsin inhibitors I, II and Des–Thr–Ser–Pro–Gln–Arg-inhibitor by air and regeneration of trypsin inhibiting activity with time. The inhibitors were reduced with dithiothreitol as described in the text. For reoxidation, the reduced inhibitors (0.5 mg/ml) was dissolved in 0.02 M phosphate buffer pH 8.0 and ventilated by bubbling air through the solution. At indicated times, a sample of 0.2 ml was withdrawn, diluted to 10 ml and tested for inhibitory activity. Lowest activity was found after elution of the reduced inhibitor from the Bio Gel column (time S).

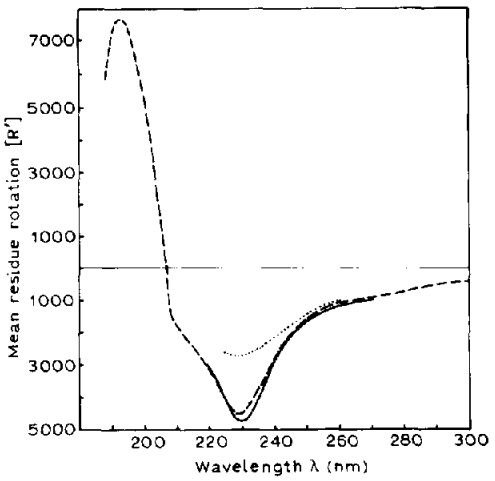

Fig. 2. Optical rotatory dispersion of pancreatic secretory trypsin inhibitor I. The curves were measured in 0.1 M phosphate buffer pH 6.0 at 20°: —— native inhibitor I, 3.15 mg/ml; . . . . . reduced inhibitor I, 0.865 mg/ml, solution 8 M in guanidine; - - - - - reduced and reoxidized inhibitor I, 3.04 mg/ml.

in 8 M guanidine and of the air reoxidized material are shown in fig. 2. The native and reoxidized inhibitors exhibit nearly identical optical rotatory dispersions typical for proteins. A deep trough was found at 230 nm, which was almost absent in the denatured and reduced inactive inhibitor. The reduced inhibitor exhibits a less ordered structure as was obvious from the depth of the trough of the optical rotatory dispersion with $[R']_{red.} = -2780$. The α-helix content of the proteins may be estimated from the depth of the trough [14]. For native inhibitor I a mean residue rotation of $[R']_I = -4280$ was calculated which indicated 26% α-helix content. The corresponding values for the fully reduced inhibitor after air reoxidation amounted to $[R']_{red.-reox.} = -4160$, i.e. 25% α-helix content. The absolute values calculated for α-helix content should be accepted with reservation, because they might be altered considerably by β-structure [18] and because of the general uncertainty whether it is valid to assume that a protein is a simple mixture of α-helical and random coiled regions. But it is obvious that the optical rotatory dispersions of native and reduced inhibitor I are in excellent agreement within experimental error after reoxidation of the latter. The results presented here indicate that the native secondary and tertiary structure of inhibitor I may be preferentially regenerated on oxidation after full reduction, thus restoring full biological activity. No additional template nor the complete amino acid sequence was necessary for this process. Cleavage of the first four amino acid residues (inhibitor II) from the amino terminal end had no significant influence on time and course of the process of regeneration upon reoxidation, though the reaction was slowed down slightly. It seemed likely that formation of ion pairs from the positively charged side chain of the arginine (position 5) and negatively charged residues of the dicarboxylic acid residues would render the process of correct folding and coiling more difficult. Therefore, elimination of the first five amino terminal residues including this arginin even facilitated the regeneration of the activity during reoxidation as could be demonstrated on Des–Thr–Ser–Pro–Gln–Arg-inhibitor.

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