RANDOMLY REOXIDISED SOYBEAN TRYPSIN INHIBITOR AND THE POSSIBILITY OF CONFORMATIONAL BARRIERS TO DISULPHIDE ISOMERIZATION IN PROTEINS

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Received 21 August 1975

1. Introduction

The process by which a protein folds to its native conformation is determined by both thermodynamic and kinetic constraints. The classic work of Anfinsen [1,2] demonstrated that the thermodynamic stability of the native state determines the product of folding of reduced ribonuclease. However the size of proteins and the observed time scale of refolding make it clear that a random search of all possible structures does not occur [3]. Much recent theoretical and experimental work of different kinds has shown that folding generally follows a specified sequence, the course of folding being determined by the existence of centres of nucleation [4–11]. The recent work of Creighton [12–15] has established an obligatory sequence for the formation of disulphide bonds during the oxidation of pancreatic trypsin inhibitor in native conditions; only 4 out of 15 possible isomers containing one disulphide bond, and only 4 out of 45 isomers containing 2 disulphide bonds actually form to any extent as intermediates. All this evidence shows that a large proportion of possible conformations are never sampled by an unfolded protein in native conditions; the conformational potential energy barriers are too great. But these conformations may be explored in denaturing media, and some, at least, can be 'fixed' by reoxidation in denaturing conditions, so-called 'random' reoxidation. If 'randomly' reoxidised proteins are returned to non-denaturing conditions, the 'native' potential energy surface can be explored from the 'far side' of the potential energy barriers. It is possible that these barriers inhibit conversion of the 'randomly' reoxidised proteins to the native conformation even when disulphide interchange is allowed, just as they inhibit the reverse transition in the course of normal oxidation. We provide evidence consistent with this possibility for soybean trypsin inhibitor; this protein contains only 2 disulphide bonds [16] so that structural analysis of the 'randomly' reoxidised state should not be too complex.

2. Materials and methods

Soybean trypsin inhibitor (STI), trypsin, insulin and sarcosine hydrochloride were obtained from Sigma; randomly reoxidised ribonuclease from Miles-Seravac; urea and dansyl-chloride (both Aristar grade) from BDH; gel-filtration materials from Pharmacia. Other reagents were analytical grade. Double-glass-distilled water was used throughout.

STI was dansylated by the standard procedure [17], but to limit the extent of reaction, incubation was for 1 h only, at 4°C. The dialysate was lyophilised and stored at 4°C. Samples of STI or dansyl-STI (40 mg in 2 ml) were reduced by 5 mM DTT in 10 M urea at 37°C; reduction was complete within 15 min, assayed by the method of Iyer and Klee [18]. Reduced protein was isolated with minimal reoxidation by immediate elution from G-25 Sephadex (40 × 1 cm column) in 0.1 M acetic acid, diluted to 0.4 mg/ml and reoxidised in air; various denaturing conditions were used (fig.1). The disappearance of –SH groups was followed using DTNB [19] except where the medium contained Cu^{++} ions to accelerate reoxidation [20]; in that case the absence of –SH groups was confirmed by the use of pCMB [21]. When reoxidation was complete the product was concentrated by pressure

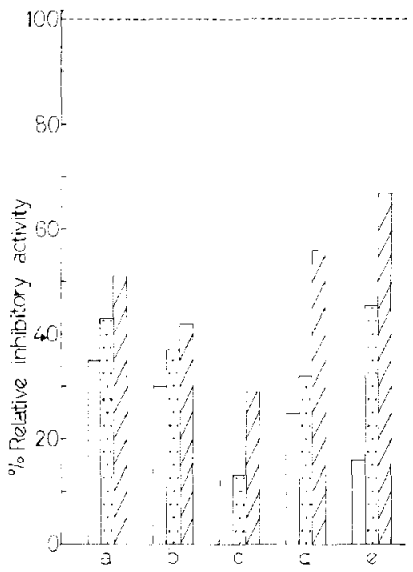

Fig.1. The three columns represent inhibitory activity assayed (i) directly, (ii) after incubation in 0.1 M Tris-HCl pH 8.0 for 20 h, (iii) after incubation as (ii) in the presence of 1 mM mercaptoethanol and 4 μg/ml protein disulphide isomerase. 'Random' reoxidation conditions: (a) 0.1 M Tris–HCl pH 8.0, 8 M urea, 0.1 M sarcosine-HCl (data are mean of 4 preparations); (b) as (a) but using dansyl-STI (2 preparations); (c) 0.1 M acetic acid, pH 3.0; (d) as (c) plus 8 M urea, 0.1 M sarcosine-HCl (3 preparations); (e) as (d) plus 0.02 mM $CuSO_4$ (2 preparations).

dialysis, adjusted to pH 3.0 with acetic acid and eluted from G-25 Sephadex (40 × 2 cm column) in 0.1 M acetic acid.

To fractionate the product, the material in 0.1 M acetic acid was concentrated further by pressure dialysis and eluted from G-100 Sephadex (40 × 2 cm column) in the same solvent.

The concentration of protein and the number of dansyl groups incorporated per molecule were calculated from absorbance measurements at 280 nm and 330 nm carried out at pH 7–8. The extinction coefficient of the dansyl group was taken as $3.36 \times 10^3 M^{-1}\ cm^{-1}$ at 330 nm and half this at 280 nm [22,23]. The absorbance of 1 mg/ml STI was 0.03 cm^{-1} at 330 nm and 0.86 cm^{-1} at 280 nm.

STI activity was determined from the inhibition of trypsin activity using benzoyl arginine ethyl ester (BAEE) as substrate [24] and following absorbance at 253 nm. Inhibition was linear with the quantity of inhibitor present, up to 90% inhibition, for native STI and for the various chemically modified preparations; reduced inhibitor was completely inactive. Measurements of trypsin activity alone (a_0), in the presence of S μg native inhibitor (a_s) and in the presence of I μg modified inhibitor (a_i) were used to calculate the % relative inhibitory activity, the activity of the modified inhibitor compared to that of an equal quantity of native inhibitor = $(a_0 - a_i)S/(a_0 - a_s)I \times 100$.

Protein disulphide isomerase (EC 5.3.4.1.) was prepared from beefliver by Gurari's modification [25] of the standard procedure [26]. The partially purified preparation after the stage of ion-exchange chromatography on CM-Sephadex C-50 was lyophilised and stored at 4°C. The further purification and enzymic characterization of this material will be discussed elsewhere. The preparation was assayed for insulin-polymerizing ability by the method of Gurari [25] and for ribonuclease reactivation by the method of Cottrell and Rabin (personal communication).

Fluorescence measurements were performed on a Perkin-Elmer MPF 3 spectrofluorimeter.

3. Results and discussion

STI which had been reduced and then reoxidised in denaturing conditions was assayed for trypsin inhibitory activity (fig.1). When the assay was performed directly on the reoxidised product, the activity was approximately 25% of that of native inhibitor. However, this activity was increased if the inhibitor was diluted into the neutral assay buffer and incubated before trypsin and the substrate were added; the increase showed first order kinetics with $t_{1/2}$ of 2 min. The inhibitory activity could be increased still further if the 'randomly' reoxidised inhibitor was incubated in the presence of mercaptoethanol and protein disulphide isomerase before assay. However, in no case was activity approaching that of native inhibitor recovered.

These findings suggest that the 'randomly' reoxidised preparations contain (1) native STI, (2) reversibly denatured material with the correct disulphide pairing, (3) protein with incorrect disulphide pairings which can be reactivated by

disulphide interchange and (4) irreversibly inactivated material. Fig.1 shows that the proportion of product in each group can be varied by the conditions of 'random' reoxidation (pH, concentration of urea, rate of oxidation etc.). The proportion of material which is active or can be activated without disulphide exchange is usually around 33%, the value which would be expected on the simple (and surely untenable) assumption that all disulphide pairing arrangements are equally probable. The proportion which can be activated by mercaptoethanol plus protein disulphide isomerase ranged from 10–25%; similar reactivation was also achieved by long periods of incubation with mercaptoethanol alone. However a proportion of the product, varying from 30% to 70%, could not revert to the active state in any of the conditions used. This suggests that in denaturing conditions, conformations can be taken up and fixed by formation of –S–S– links, which in non-denaturing conditions are separated from the native conformation by substantial potential energy barriers; this conclusion is reinforced by the fact that the size of the non-activatable fraction varies with the conditions of reoxidation.

STI, dansylated to a minimal extent, was used to investigate differences in conformation between the native, reduced and 'randomly' reoxidised states. The protein was labelled to the extent of 1.4 dansyl groups per molecule. Previous work [27] has shown that only 4 residues in STI are reactive nucleophiles, and that one of these is exceptionally reactive, so that the majority of the dansyl groups are probably attached to a specific residue. This small extent of dansylation produces little loss of inhibitory activity. The behaviour of dansyl-STI on reduction, reoxidation and reactivation is very similar to that of the unlabelled protein (compare figs.1a and 1b). The fluorescence emission spectra show that the gross conformation of the reduced protein is substantially different from that of native dansyl-STI; intrinsic protein fluorescence almost doubles, probably as a result of reduced energy transfer to the dansyl group, and the dansyl fluorescence increases even more strikingly (fig.2). 'Random' reoxidation returns the fluorescence towards that of native dansyl-STI (as would be expected since more than 30% of the product of this oxidation is fully active inhibitor), but the net dansyl fluorescence of the 'randomly' reoxidised

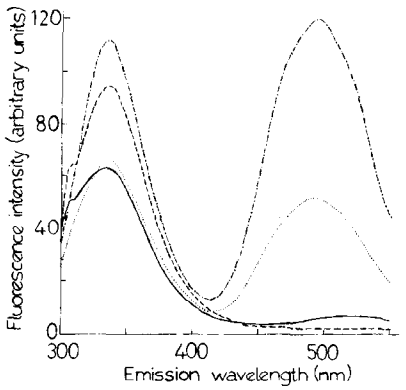

Fig.2. Fluorescence emission spectra recorded at room temperature with excitation at 280 nm; intensity corrected for sample absorbance. Samples contained 80–110 μg/ml protein in 0.1 M Tris–HCl pH 7.0. Native STI (- - -); dansyl-STI (———); reduced dansyl-STI (- . - . -); 'randomly' reoxidised dansyl-STI (. . . .).

product is still considerably enhanced. Incubation of this material with mercaptoethanol and protein disulphide isomerase decreases the dansyl fluorescence still further (not shown), which is consistent with the observed conversion of a small proportion of the product to the native state by disulphide interchange (fig.1b).

Our hypothesis that the irreversibly inactivated material is prevented by conformational potential energy barriers from returning to the active state is not inconsistent with the use of the enzyme, protein disulphide isomerase, to catalyse disulphide interchange. While this enzyme lowers the activation energy of the chemical reaction, it certainly cannot lower the potential energy barriers to conformational change in its substrates. In fact we have evidence which suggests that the enzyme may act chemically on the irreversibly inactivated material. The preparation of enzyme is very active in reactivating 'randomly' reoxidised ribonuclease and in polymerizing insulin; both these activities are substantially inhibited by the addition of 'randomly' reoxidised STI. It is possible that irreversibly inactivated 'randomly' reoxidised STI is an alternative substrate for protein disulphide isomerase, but one which cannot be converted to the active form, so that its presence is expressed only in the inhibition of the action of the enzyme on other substrates. The fact that 'randomly' reoxidised

ribonuclease and lysozyme inhibit disulphide bond interchange between glutathione and insulin has recently been interpreted in a similar way [28].

The reactivation studies show that 'randomly' reoxidised STI is structurally complex and is not a simple 1:1:1 mixture of three possible disulphide linked isomers. Detailed structural characterization is obviously called for. We have shown by gel-filtration that the material is heterogeneous in molecular weight, confirming the findings of Steiner [29]. Reoxidation at concentration down to 5 μM protein leads to product mixtures in which almost 50% of the material is of higher molecular weight than monomer STI. We have tested both monomer and polymer components for activity and susceptibility to reactivation. Both components showed some native activity (fraction 1), increases on control incubation and incubation with protein disulphide isomerase (fractions 2 and 3) and a residue of irreversibly inactivated material (fraction 4).

The findings above are consistent with recent work on the limitation on conformational freedom during reoxidation (e.g. of pancreatic trypsin inhibitor) but contrast with some common assumptions mainly based on work on ribonuclease. For ribonuclease, there is good evidence that random reoxidation generates a product practically devoid of activity, which can be reactivated in good yield by disulphide interchange [1,2]; the implication is that no large conformational barriers exist between the native state and the alternative disulphide-bonded isomers which form during reoxidation in denaturing conditions. ('Randomly' reoxidised ribonuclease has never been characterized in detail and may consist of only a small fraction of the 105 potential disulphide linked isomers). This absence of conformational barriers is confirmed by detailed analysis of the process of reoxidation in native conditions [30]. The time course of disappearance of –SH groups is very much faster than the time-course of conformational changes monitored by intrinsic probes, or that of appearance of activity. It seems that in ribonuclease, reoxidation occurs rapidly and there is then slow disulphide interchange leading from various fully oxidised isomers to the native state (a process analogous to that which occurs when 'randomly' reoxidised ribonuclease is reactivated in the presence of protein disulphide isomerase).

By contrast, the work on pancreatic trypsin inhibitor [12–15] demonstrates that incorrect fully reoxidised isomers never form during the reoxidation of this protein in native conditions, and that only a small proportion of possible intermediates are explored. It is not surprising that proteins differ in this respect. Soybean trypsin inhibitor may resemble pancreatic trypsin inhibitor in having an obligatory order for the formation of disulphide bonds. This is suggested by the fact that in both proteins one disulphide bond can be reduced selectively with little effect on the protein's properties [15,31]; in pancreatic trypsin inhibitor, this labile bond is the one which must form last in the normal folding scheme [15]. In any case, there is no objection in principle to the proposal made here, namely that reoxidation in denaturing conditions can lead to stabilization of conformers which isomerize at a negligible rate to the active conformation, even in native conditions with catalysis of disulphide interchange. The data on randomly reoxidised STI are consistent with this proposal.

Acknowledgement

We acknowledge with thanks the support of the Science Research Council.

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