Controlling the speed of hirudin folding

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The folding of hirudin undergoes an initial stage of non-specific packing, followed by consolidation (re-organization) of partially packed intermediates to attain the native structure [Chatrenet and Chang (1993) *J. Biol. Chem.* **268**, 20988–20996]. Non-specific packing leads to the formation of scrambled hirudins as folding intermediates. A systematic study was carried out to

search for conditions which would selectively control and enhance the processes of packing and consolidation. It is demonstrated here that under optimized conditions, including the use of cystine/cysteine and protein disulphide isomerase, the folding of hirudin *in vitro* can be achieved quantitatively within 30 s.

INTRODUCTION

Correct folding of genetically engineered proteins is a major issue in protein science (King, 1986; Dill, 1990; Richards, 1991). The problem is particularly acute in the case of cystine-containing proteins (Freedman, 1984), which often need to be denatured and refolded to become active (Buchner et al., 1992; Fischer et al., 1993; Vandebroek et al., 1993). Disulphide formation is an event of post-translational modification. It follows the pathway of protein folding and usually occurs faithfully *in vivo* (Freedman, 1984; Gething and Sambrook, 1992). However, in renaturation experiments *in vitro*, many proteins are recovered in poor yields and some do not fold into native structures at all (Cleland et al., 1992; Fischer et al., 1993). To overcome this problem a number of compounds, such as the mixture of reduced/oxidized glutathione (Saxena and Wetlaufer, 1970; Hantgan et al., 1974; Orsini et al., 1975; Creighton, 1986) and protein disulphide isomerase (PDI) (Epstein et al., 1963; Morin and Dixon, 1985; Freedman, 1989), have been routinely used to promote the formation of disulphides. But despite their widespread use, the precise mechanism of their function remains undefined and most of their applications have been conducted in a manner of trial and error (Lyles and Gilbert, 1991; Lundstroem et al., 1992). It is therefore not surprising that the efficiencies of folding *in vitro* documented so far are nowhere near those observed *in vivo* (Gething and Sambrook, 1992).

Hirudin is a thrombin-specific inhibitor originating from leech *Hirudo medicinalis* (Markwardt, 1990). The inhibitor contains two functional domains (Rydel et al., 1990). The core domain (49 amino acids) (Chang, 1990) is stabilized by three disulphides and requires proper folding to maintain its biological function. Recently, the *in vitro* folding pathway of hirudin core domain has been elucidated by structural analysis and stop/go folding experiments of acid-trapped intermediates (Chatrenet and Chang, 1992, 1993). The results demonstrate that folding of hirudin is initiated by non-specific packing, followed by consolidation (re-organization) of partially packed intermediates to reach the native structure. The course of packing involves a sequential flow of the unfolded sample (R) through equilibrated 1-disulphide isomers (I) and equilibrated 2-disulphide isomers (II) to equilibrated 3-disulphide (scrambled) species (III). The

process of consolidation is characterized as scrambled species re-organize by reshuffling the non-native disulphides to attain the native species (N). The data (Chatrenet and Chang, 1993) also reveal that the relative speeds of packing and consolidation hinge upon the folding conditions and can vary substantially. For instance, it has been shown that packing (disulphide formation) is promoted primarily by GSSG, whereas consolidation (disulphide reshuffling) requires GSH (or 2-mercaptoethanol) as the catalyst. These findings point out an effective route for improving the efficiency of hirudin folding and permit a rational approach for the design of desirable folding conditions.

In this report, a systematic study has been performed in order to find optimized conditions that would selectively, as well as cooperatively, expedite the processes of packing and consolidation. The aim is to explore the factors limiting the speed of hirudin folding *in vitro* and to attempt to imitate the efficiency of folding of disulphide-containing proteins *in vivo*.

EXPERIMENTAL

Materials

Folding was carried out with the hirudin core domain (residues 1–49). It was purified to greater than 99% by the method described previously (Chang, 1990). Reduced glutathione (GSH, G-42514), oxidized glutathione (GSSG, G-4501), cysteine (Cys, C-7755), cystine (Cys–Cys, C-8755) and oxidized dithiothreitol (DTT) (*trans*-4,5-dihydroxy-1,2-dithiane, D-3511) were obtained from Sigma. 2-Mercaptoethanol and DTT are products of Merck. PDI (no. 7318) was purchased from Takara, Kyoto, Japan.

Preparation of scrambled hirudins (III)

Two methods were used for the preparation of scrambled hirudins.

Method 1

Fully reduced/denatured hirudin (R) (0.25 mg/ml) was allowed to refold in NaHCO₃ buffer (0.1 M, pH 8.3) for 24 h at 23 °C. At the end of the folding, 0.1 M iodoacetic acid was introduced. The sample was kept at 23 °C for another 20 min, lyophilized and

Figure 1 Purification and characterization of scrambled hirudins (III)

Scrambled hirudins prepared by methods 1 and 2 (see the Experimental section) were purified by h.p.l.c. (a). Their molecular mass (b) and disulphide content (c) were determined by laser-desorption ionization m.s. and the dimethylaminoazobenzenesulphonyl chloride method (Chang and Knecht, 1991) respectively. Scrambled hirudins obtained from method 1 contain three intact Cys–Cys (no detectable free cysteine) (c, top) and display a single molecular mass of 5059 (b, top). Those prepared by method 2, however, are contaminated by two minor species (b, bottom); each constitutes about 2–3%. The one with a molecular mass of 5671 corresponds to the structure of two intradisulphides and two mixed disulphides with glutathione. The molecular mass of 5423 is consistent with the structure of two intradisulphides, one mixed disulphide with glutathione and one free cysteine (carboxymethylated; CmCys). Two internal standards, with molecular masses of 3417 (calcitonin) and 8132 (eglin c), were used for calibration. The existence of the free cysteine (2.5%) was further confirmed by amino acid analysis (c, bottom, see arrow indicating CmCys).

(III) was purified by h.p.l.c. (Figure 1, top-left). The yield was $75 \pm 5\%$.

Method 2

(R) (0.7 mg/ml) was allowed to refold for 10 min in NaHCO_3 buffer (0.1 M, pH 8.3) containing 2 mM GSSG. The sample was then carboxymethylated with 0.1 M iodoacetic acid (for 10 min), lyophilized and (iii) was isolated by h.p.l.c. (Figure 1, bottom-left). The yield was $70 \pm 5\%$. Procedures for the preparation of (R) as the starting material for folding and conditions for h.p.l.c. purification of scrambled hirudins are described below.

Structural characterization of scrambled hirudins

Scrambled hirudins were analysed for their molecular mass using laser-desorption ionization mass spectrometry (Boernsen et al., 1990) and their amino acid compositions by the dimethylaminoazobenzenesulphonyl chloride method (Chang and Knecht, 1991), which allows direct quantification of disulphide bridges.

Folding experiments

Packing

The starting material of folding, fully reduced/denatured hirudin (R), was prepared by the following method: native hirudin core domain (2 mg/ml) was dissolved in Tris/HCl buffer (0.5 M, pH 8.5) containing 5 M guanidinium chloride and 30 mM DTT. Reduction and denaturation were carried out at 23 °C for 90 min. To initiate the folding, the sample was passed through a PD-10 column (Pharmacia) equilibrated in 0.1 M NaHCO_3 buffer (pH 8.3). Desalting took about 1 min and (R) was collected in 1.1 ml. It was immediately distributed and diluted to a final concentration of 70 μM containing selected concentrations of

GSSG, Cys–Cys, oxidized DTT or PDI. Folding intermediates were trapped in a time-course manner by mixing aliquots of the sample with an equal volume of 4% (v/v) trifluoroacetic acid and were subsequently analysed by h.p.l.c. using the following conditions: the column was Vydac C-18 for peptides and proteins, solvent A was water containing 0.1% trifluoroacetic acid and solvent B was acetonitrile/water (9:1, v/v) containing 0.1% trifluoroacetic acid. The linear gradient was 16–30% solvent B in 50 min. The flow rate was 1 ml/min.

Consolidation (re-organization of scrambled hirudins)

Experiments on consolidation were initiated with purified scrambled hirudins (III). They were dissolved in NaHCO_3 buffer (0.1 M, pH 8.3, protein concentration 70 μM) in the presence of various concentrations of Cys, GSH, 2-mercaptoethanol, DTT or PDI. The reaction was carried out at 23 °C and was stopped at different time intervals by mixing aliquots of the sample with an equal volume of 4% (v/v) trifluoroacetic acid, followed by h.p.l.c. analysis.

Analysis of the efficiencies of packing and consolidation

The folding of hirudin involves complex kinetics and an exceedingly large number of folding intermediates, which include nearly all possible 1-, 2- and 3-disulphide (scrambled) isomers (Chatrenet and Chang, 1993). However, the process of packing and consolidation can be simplified and treated as a consecutive two-stage reaction:

Figure 2 Three different states of the compositions of the folding intermediates of hirudin

(a) Intermediates which remain predominantly as a mixture of (I) and (II). (b) Intermediates which consist of (I), (II) and (III). (c) Intermediates which are trapped exclusively as (III). This figure illustrates how different species of intermediates were quantitatively measured during the folding of hirudin. (I), (II) and (III) stand for 1-disulphide, 2-disulphide and 3-disulphide (scrambled) intermediates respectively (for their structural analysis see Chatrenet and Chang, 1993). It is difficult, on the basis of the h.p.l.c. pattern alone, to quantify (I) and (II) individually because of their heterogeneity and extensive overlapping. Even (R) is co-eluted with some 1-disulphide species. However, (III) can be determined with confidence. Scrambled species always exist in equilibrium along the folding process and part of (III) does not overlap with any other disulphide species. Fractions b, c, d, e, f, g and h are present in equilibrium with the molar ratio of 102:100:34:17:28:27:19 (average from 12 samples with S.D. of < 5%). Knowing the recovery of the non-overlapping fractions (for example, c, d, e or f) would consequently allow us to calculate the total yield of (III). In the case of sample (B), the yield of (R + I + II) could be determined eventually by subtracting (III) and (N) (native species) from the total peak area.

where (I), (II) and (III) stand for 1-disulphide, 2-disulphide and scrambled 3-disulphide species respectively. In such a process, the rate of the increase of (III) is the difference between the rate of its formation and that of its consumption to form (N). Thus the level of accumulation of scrambled hirudins along the folding pathway is dependent upon the relative rate of packing and consolidation, which in turn are regulated by applied redox potential (see results below). It is relevant to point out that, by adopting this simplified kinetic model, we take the scrambled species as a landmark to determine the speed of packing. This is because scrambled species represent the most advanced state of packing and can be quantitatively analysed with accuracy.

For the evaluation of redox agents which influence the speed of packing and consolidation, the recoveries of three groups of hirudin derivatives, (R + I + II), (III) and (N) were quantitatively analysed during the folding process. (R + I + II) represents the combined yield of 0-disulphide starting material, 1- and 2-

disulphide intermediates. (R + I + II) overlaps partially with (III) (see Figure 2), but recoveries of these two groups of hirudin species can be determined through the non-overlapping fractions of (III) (e.g. fractions d, e, f and g, see Figure 2c) because scrambled hirudins exist in equilibrium at constant molar ratio (see legend of Figure 2).

Anti-amidolytic and anticoagulant assays of refolded hirudins

Anti-amidolytic activities of refolded samples were measured by their ability to inhibit human α -thrombin from digesting Chromozym TH (Boehringer-Mannheim). The reaction was carried out at 23 °C in 67 mM Tris/HCl buffer, pH 8.0, containing 133 mM NaCl and 0.13 % poly(ethylene glycol) 6000. The rate of digestion was followed at 405 nm for a period of 2 min. The concentration of substrate was 200 μ M. The concentration of α -thrombin was adjusted between 2.5 and 25 nM.

The anticoagulant activities of refolded samples were determined using a Coagulometer KCl (from Amelung GmbH, Lemgo, Germany). The assay was carried out at 23 °C in the same Tris/HCl buffer in a total volume of 500 μ l. α -Thrombin (150 nM) was incubated with different concentrations of hirudin samples in the Tris/HCl buffer for 2 min. Recording of the clotting time was started on mixing 100 μ l of the incubated sample with 400 μ l of fibrinogen solution (2 mg/ml). Under these conditions, the clotting time of the control sample (without added hirudin sample) was 19 ± 1 s.

RESULTS AND DISCUSSION

Preparation of scrambled hirudins

Scrambled hirudins were prepared for the purpose of analysing the efficiency of consolidation. They can be produced in good yield (65–80 %) by the following methods: (a) halting the process of consolidation (method 1) and (b) accelerating the process of packing (method 2). In method 1 conversion of scrambled hirudins to the native structure requires the action of free thiols as catalysts. When hirudin was allowed to fold in buffer alone, free cysteines of 0-, 1- and 2-disulphide intermediates serve as the thiol catalyst during the early phase of folding. As the folding advances, more cysteines become involved in the disulphide pairing and less are available as thiol catalysts, therefore scrambled species accumulate and are trapped. In method 2 the rate of packing is greatly enhanced in the presence of GSSG or cystine (Cys–Cys). This leads to the accumulation of a high concentration of scrambled species along the folding pathway (see the next section).

Scrambled hirudins prepared by these two methods are indistinguishable, as judged by their h.p.l.c. patterns (Figure 1, left-hand panel). However, analysis of their molecular mass and amino acid composition reveals that the sample prepared in the presence of GSSG (2 mM) contains about 3–4 % of species which form a mixed disulphide with glutathione (Figure 1, middle panel). The content of these minor species exhibits a positive function to the concentration of GSSG employed in the sample preparation (results not shown), suggesting that mixed disulphide species and scrambled species exist in equilibrium. The mixed disulphide species also catalyse the conversion of scrambled hirudins into the native structure. Thus scrambled hirudins prepared by method 2 are unsuitable for long-term storage. By contrast, those produced by method 1 are free of contaminants and completely stable in the alkaline buffer at room temperature for up to 72 h. Only the scrambled hirudins produced by method 1 were used for subsequent studies.

Figure 3 Acceleration of the packing process by Cys-Cys and GSSG

(a) Original chromatograms of five selected folding experiments. A control experiment carried out in the buffer alone and four folding experiments performed in the presence of 0.5 mM and 2 mM of GSSG and Cys-Cys are presented here. All 2 min samples are underlined by the relative locations of overlapping (I), (II) and (III). (N) is the correctly folded native structure. The open arrow, indicating the position of (R), is marked on each chromatogram to facilitate the comparison of the speed of the flow of intermediates. (b) Quantitative analysis of (N), (III) and (R + I + II) along the folding pathways mediated by different concentrations of GSSG and Cys-Cys. In the control experiment (see also original chromatograms), the intermediates remain mostly (> 97%) as a mixture (I) and (II) even after 90 min of folding. GSSG and Cys-Cys both have significant influence in promoting the speed of packing [flow of the intermediates to form (III)]. In the presence of 2–4 mM Cys-Cys, the only detectable intermediates after 2 min of folding are scrambled species.

Packing: Cys-Cys is about 10-fold more potent than GSSG in enhancing the process of hirudin packing

During the folding of hirudin, the speed of disulphide formation and the process of packing are drastically accelerated in the presence of GSSG or Cys-Cys. Packing leads to the accumulation of scrambled hirudins. Therefore, under these conditions, scrambled species become the most dominant folding intermediates and conversion of the scrambled species into the native structure (consolidation) becomes the major rate-limiting step of hirudin folding (Figure 3). For instance, in the presence of 2 mM Cys-Cys, the only detectable intermediates after 2 min of folding are scrambled species, but it takes longer than 90 min for their conversion into (N). The enhancement is dependent upon the concentration of GSSG and Cys-Cys. However, both reagents exhibit a narrow range of optimum concentrations, which is about 2–4 mM. At higher concentrations, these reagents inhibit the subsequent stage of consolidation. Judging from their ability to promote the accumulation of scrambled species, Cys-Cys is about 8–10-fold more potent than GSSG in enhancing the packing of hirudin folding. An additional advantage of the Cys-Cys system is that it permits a comparably more active consolidation, which is catalysed by trace amounts of mixed disulphides and free cysteine that exist in the state of equilibrium. These differences can be explained by the relative redox potential

(E_o') of the GSH/GSSG (–0.24 V) and Cys/Cys-Cys (–0.22 V) systems (Rost and Rapoport, 1964; Jocelyn, 1967). A thermodynamically favoured GSSG is less potent at promoting the disulphide formation of proteins.

Oxidized DTT exhibits no detectable influence on the course of packing. This has been observed at concentrations ranging from 0.1 mM to 10 mM (Figure 4a). The inability of oxidized DTT to promote the disulphide formation of hirudin is not surprising, as its six-member ring is energetically favoured over the mixed disulphide (DTT has a redox potential of –0.33 V at pH 7 and 25 °C) (Cleland, 1964). This is also evident from the folding experiments of ribonuclease A. Despite the application of 100 mM oxidized DTT, the folding of reduced/denatured ribonuclease A remains extremely sluggish (Rothwarf and Scheraga, 1993). In the case of chymotrypsinogen (Orsini et al., 1975), oxidized DTT cannot generate native protein at all.

PDI does not promote the process of packing, instead it functions like a free thiol, such as 2-mercaptoethanol. In the 2-mercaptoethanol-assisted hirudin folding, scrambled hirudins do not appear in significant concentrations (Chatrenet and Chang, 1993). This is because the role of 2-mercaptoethanol is to facilitate the conversion of scrambled species into the native structure (consolidation). Under these circumstances, the process of packing ends up as the major rate-limiting step of hirudin folding and 1- and 2-disulphide species become well-populated folding inter-

Figure 4 Folding of hirudin mediated by oxidized DTT and PDI

(a) (R) was allowed to refold in NaHCO_3 buffer alone (control sample) or in the presence of 0.5 mM, 2 mM or 10 mM of oxidized DTT. After 15 min of folding, the samples were trapped with acid and analysed by h.p.l.c. (b) (R) was allowed to refold in NaHCO_3 buffer containing 10 μM PDI. Folding intermediates were acid-trapped in a time-course manner and analysed by h.p.l.c. Two minor peaks marked by stars are derived from PDI.

mediates. The patterns of intermediates obtained from the PDI-mediated folding are presented in Figure 4(b). Along the entire course of folding, more than 95% of the folding intermediates

remain as a mixture of 1- and 2-disulphide species. These results bear similarity to those mediated by 2-mercaptoethanol [see Figure 2 of Chatrenet and Chang (1993)]. However, PDI appears to be far more effective than 2-mercaptoethanol in promoting the process of consolidation. In the presence of 10 μM PDI, folding of hirudin is complete within 80–90 min (Figure 4). By contrast, it requires 10–11 h for the completion of folding in the presence of 100 μM 2-mercaptoethanol. It should be mentioned (and will be shown in Figure 6) that as a reagent for catalysing the consolidation, PDI needs to be activated by free thiols. In the present case it is most likely that PDI has been activated by free thiol groups of reduced hirudin.

Consolidation: the role of PDI is to promote the process of consolidation

Consolidation of scrambled hirudins to attain the native conformation requires reshuffling of non-native disulphides. This can be catalysed by free thiols (Haber and Anfinsen, 1962) and several thiol-containing reagents have been evaluated here. These include cysteine, GSH, 2-mercaptoethanol, DTT and PDI. The analysis was initiated with purified scrambled species. The results are summarized and presented in Figures 5 and 6. A number of important observations can be concluded from these studies. (1) The effectiveness of thiol reagents in promoting consolidation is apparently related to their redox potential. The order of efficacy is as follows: cysteine (-0.22 V) > GSH (-0.24 V) > 2-mercaptoethanol > DTT (-0.33 V). (2) Each thiol reagent (except for DTT) displays an optimum concentration (Figure 5). It is about 2–3 mM for Cys, 1–2 mM for GSH and 1 mM for 2-mercaptoethanol. (3) At low concentrations of thiol reagents, disulphides reshuffle and consolidation proceeds without detectable 1- and 2-disulphide species forming as the intermediates.

Figure 5 Promotion of the process of consolidation by thiol-containing agents

(a) Chromatograms were obtained from experiments performed in the presence of 0.3 mM (top), 3 mM (middle) and 10 mM (bottom) GSH. The location of (III) is underlined. Open arrows indicate the elution position of (R). Noted that in the presence of 3 mM and 10 mM GSH, part of (III) is converted into (R+I+II) then slowly swung back to (N). (b) Quantitative analysis of (N), (III) and (R+I+II) during the process of consolidation catalysed by cysteine, GSH and 2-mercaptoethanol (2-ME). Scrambled hirudins are completely stable in the buffer alone (control sample).

Figure 6 Effect of PDI on the consolidation of scrambled hirudins

Scrambled hirudins (70 μ M) were dissolved in NaHCO_3 buffer (0.1 M, pH 8.3) containing PDI (10 μ M) alone (top panel), cysteine (1 mM) alone (middle panel), or a mixture of PDI (10 μ M) and cysteine (1 mM) (bottom panel). PDI alone has no effect. However, in the presence of cysteine, PDI displays an additive potency in promoting the conversion of (III) into (N). Hatched bars and solid bars represent the yields of (III) and (N) respectively. Similar results have been observed in the presence of 4 μ M PDI.

This has been observed, for example, with 0.3 mM cysteine or GSH. (4) In the presence of high concentrations of thiol reagents, part of the scrambled hirudins are converted into the native species through a Yo-Yo mechanism. They are first reduced to the state of (R+I+II), then slowly swing back to (N). This phenomenon is particularly visible with 10 mM cysteine and 3 mM GSH and 2-mercaptoethanol. (5) Unlike cysteine, GSH or 2-mercaptoethanol, DTT fails to catalyse any measurable conversion of (III) into (N). Instead, it reduces scrambled hirudins and pushes (III) only toward the direction of (R+I+II), which occurs at DTT concentrations ranging from 30 μ M to 3 mM (results not shown). The difference of the redox potentials between DTT (-0.33 V) (Cleland, 1964) and cysteine (-0.22 V) accounts for this nearly irreversible reaction.

PDI alone has no effect on the consolidation of scrambled hirudins, which remain completely stable in alkaline buffer containing 2 μ M or 10 μ M PDI (Figure 6, top panel). However, in the presence of free thiols, PDI displays an additive effect in enhancing the process of consolidation. For instance, with 1 mM cysteine, PDI (10 μ M) accelerates the initial rate of the recovery of (N) by 2-fold. Similar enhancement has also been observed when consolidation was performed in the presence of 0.1 mM cysteine and 10 μ M PDI. In addition, activated PDI is likely to be more potent than cysteine at an equivalent concentration. PDI contains functional units homologous to thioredoxin, which possesses a redox potential (-0.20 V) (Moore et al., 1964) more favourable than that of cysteine in promoting the recovery of (N) from (III).

Figure 7 Fine tuning of the Cys/Cys-Cys composition in order to search for an optimized redox potential to facilitate hirudin folding

Hirudin was reduced and denatured as described in the Experimental section. To initiate folding, the sample was passed through a PD-10 column equilibrated in the Tris/HCl buffer (0.2 M, pH 8.5). (R) was recovered and reconstituted in a solution containing 4 M NaCl and various concentrations of the Cys/Cys-Cys mixture. Folding intermediates were acid-trapped and analysed by h.p.l.c. (a), 4 mM cysteine/2 mM Cys-Cys; (b), 3 mM cysteine/3 mM Cys-Cys; (c), 2 mM cysteine/4 mM Cys-Cys. Under these folding conditions, the only detectable intermediates (after 1 min) are scrambled species. Solid bars, hatched bars and open bars represent the recoveries of (N), (III) and (R+I+II) respectively. In this case, the open bars at $t = 0$ represent exclusively (R).

Folding of hirudin can be achieved within 30 s *in vitro*

The results obtained from the analysis of packing and consolidation suggest that efficient folding of hirudin requires optimized concentrations of the cysteine and Cys-Cys mixture. Experiments ($n = 16$) to study the folding of hirudin were performed in the presence of various concentrations (1 mM to 10 mM) and molar ratios (0.5 to 5) of the Cys/Cys-Cys mixture. The combination of 4 mM of cysteine and 2 mM of Cys-Cys was found to produce the best enhancing effect. Under these conditions, folding of hirudin was completed within 5 min (Figure 7). Comparison between the Cys/Cys-Cys and GSH/GSSG systems under equivalent conditions also confirms that the former one is about 5–6-fold more potent in promoting the formation of native hirudin (results not shown). By combining the optimized Cys/Cys-Cys redox potential with PDI, it was possible to improve the speed of hirudin folding further. In the presence of 50 μ M PDI, the completion of hirudin folding can be achieved within 30 s (Figure 8).

Judging by the anti-amidolytic activity and anticoagulant activity, the refolded hirudin was indistinguishable from the native one. Both samples exhibited a dissociation constant of 70 ± 10 nM toward thrombin binding. In the anticoagulant assay, both samples also displayed nearly identical potency. The S.D. of the clotting time, observed at hirudin concentrations ranging from 100 nM to 1 μ M, was within $\pm 4\%$.

Figure 8 Accelerated pathway of hirudin folding

(R) was allowed to refold in Tris/HCl buffer (0.2 M, pH 8.5) containing NaCl (4 M), Cys/Cys–Cys (4 mM/2 mM) and PDI [10 μ M (a) and 50 μ M (b)]. Folding intermediates were trapped with acid and analysed by h.p.l.c. Open arrows indicate the position of (R). Two minor peaks marked by stars are derived from PDI. In the presence of 50 μ M PDI, folding was virtually completed within 30 s. It should be pointed out that this time scale does not include that required for desalting (about 1 min). In a separate control experiment, it has been shown that the starting material (after desalting) consisted of only (R) (75%) and (I + II) (25%).

Concluding remarks

It has been demonstrated here that the speed of hirudin folding and the compositions of its folding intermediates can be manipulated by adjusting the redox potential, which influences the processes of packing and consolidation. It is found that the Cys/Cys–Cys redox system is superior to that of GSH/GSSG and the major role of activated PDI is to promote the process of consolidation. Knowledge obtained from these studies has enabled us to shorten the time required for folding of hirudin *in vitro* from 10 h (Chatrenet and Chang, 1993) to 30 s. This efficiency of folding *in vitro* does not differ significantly from what occurs *in vivo*, which has been shown to be accomplished within 2 min (Freedman, 1984).

Finally, one relevant question which will undoubtedly be raised is whether the folding mechanism (Chatrenet and Chang, 1993) and the control of folding efficiency observed in hirudin represents a unique case or a general phenomenon. There are few proteins, except for bovine pancreatic trypsin inhibitor (BPTI) (Creighton, 1978; Weissman and Kim, 1991) and ribonuclease (Hantgan et al., 1974; Scheraga et al., 1984; Pigiet and Schuster, 1986), which have had their folding mechanisms investigated in great detail at the molecular level. Of those which were set to refold in the presence of GSH/GSSG or PDI (Saxena and Wetlaufer, 1970; Pigiet and Schuster, 1986; Lyles and Gilbert, 1991), none has claimed to achieve efficiency comparable with those achieved in the cell. With only limited examples, it is premature to make any generalization. Nonetheless, comparisons can be drawn from the existing models. The two-stage model of hirudin folding is distinguished from that observed in BPTI

folding (Creighton, 1978; Weissman and Kim, 1991). The difference does not lie in the extent of complexity of the folding intermediates. On the basis of the reported data (Weissman and Kim, 1991) the folding intermediates of BPTI are just as heterogeneous as those of hirudin, and they are also present in a state of equilibrium. The main distinction is that scrambled 3-disulphide BPTIs have not been found to exist along its folding pathway. However, the folding of pro-BPTI (Weissman and Kim, 1992) (BPTI with an N-terminal leader sequence of 13 amino acids) appears to bear a close resemblance to hirudin. When reduced/denatured pro-BPTI refolds in the presence of GSH/GSSG (2 mM:0.5 mM), a group of well-populated, scrambled pro-BPTIs are visible as folding intermediates. The h.p.l.c. pattern of scrambled pro-BPTIs appears strikingly similar to that of scrambled hirudins.

On the other hand, the folding mechanism of hirudin is essentially accordant with that of ribonuclease (Hantgan et al., 1974; Pigiet and Schuster, 1986). Both engage an exceedingly large number of folding intermediates (Scheraga et al., 1984; Rothwarf and Scheraga, 1993; Chatrenet and Chang, 1993) and in both cases disulphide formation occurred much more rapidly than the recovery of their biological activity (Anfinsen et al., 1961; Chatrenet and Chang, 1992). When reduced/denatured ribonuclease is permitted to refold in the buffer alone, only 10% of molecules make their way to reach the native structure (Pigiet and Schuster, 1986), the remaining 90% are trapped as inactive species, which presumably are scrambled ribonuclease. Like hirudin, those trapped ribonuclease species can be eventually converted into the native ribonuclease by addition of free thiols in the folding buffer (Haber and Anfinsen, 1962). A two-step mechanism has also been demonstrated during the renaturation of ribonuclease (Pigiet and Schuster, 1986), in which an initial stage of disulphide formation is promoted by GSSG, followed by disulphide reshuffling catalysed by reduced thioredoxin.

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