Volume 336, number 1, 53–56 FEBS 13307 December 1993

Production of disulfide-linked hirudin dimer by in vitro folding

Jui-Yoa Chang*, Hugo Grossenbacher, Bernd Meyhack, Walter Maerki

Pharmaceuticals Research Laboratories, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

Received 13 September 1993; revised version received 15 October 1993

A simple process of in vitro folding has been developed for the preparation of hirudin dimer. A variant of recombinant hirudin with Asp^{33} replaced by Cys was expressed in yeast and isolated by HPLC. Crude Cys^{33}-hirudin contains heterogeneous products that are made of one species of primary sequence. They were together reduced/denatured, and allowed to re-fold in the sodium bicarbonate buffer (pH 8.3) alone. Active, homogeneous Cys^{33}-hirudin monomer folded spontaneously with a first order rate constant of 0.05 ± 0.01 min^{-1}, followed by the oxidation of two Cys^{33} to produce the pure dimer. The folding yield was 90%. On an equal weight basis, both Cys^{33}-hirudin monomer and the dimer exhibit thrombin inhibitory activity comparable to that of wild-type hirudin. Due to the presence of an extra cysteine, the folding of active hirudin monomer (formation of three native disulfides) was accelerated by at least 12-fold.

Hirudin dimer; Protein folding; Disulfide-linked hirudin dimerization

1. INTRODUCTION

Hirudin (containing 65 amino acids and three disulfide bonds) is a thrombin-specific inhibitor originally isolated from leech *Hirudo medicinalis* [1]. Recombinant hirudin is now produced in large quantities from yeast [2] and is under development as an antithrombotic drug [3]. Recombinant hirudin has a relatively short half-life and is rapidly excreted from the human body [4]. In order to evaluate the application of longer active hirudins, higher molecular weight forms, such as a hirudin dimer, have been synthesized. Such modified hirudins can be expressed and fold naturally if proper inter-disulfide bonds are created to link the monomers. Hirudin contains two functional domains which bind to independent sites of α-thrombin [5–7]. A compact N-terminal core domain binds to the catalytic site of thrombin [8] and a disordered C-terminal tail which is complementary to the exosite (fibrinogen recognition site) of the enzyme [9,10]. Linking two monomers through the C-terminal region is not desirable as nearly every amino acid residue inside this domain is required for full potency [8,9]. Within the core domain, however, there is a flexible loop (residues 31–36) [11,12] which is located on the surface and is distant from the thrombin binding site. Amino acid sequence data have revealed that mutations of hirudin variants [13] within this loop do not significantly alter the activity of hirudin. Most importantly, dimer linkage via this loop would probably impose only a minimum interference upon the binding of the dimer to two separate thrombins. This report describes the in vitro folding and dimerization of yeast-expressed Cys^{33}-hirudin, a mutant of desulfato hirudin variant 1 (HV 1) [14].

*Corresponding author. Fax: (41) (61) 696 6174.

Abbreviations: HV1, recombinant hirudin variant 1; BPTI, bovine pancreatic trypsin inhibitor.

2. EXPERIMENTAL

2.1. *Isolation of crude Cys^{33}-hirudin*

The expression in yeast (*Saccharomyces cerevisiae*) of Cys^{33}-hirudin, a mutant of the recombinant HV 1, with cysteine replacing aspartic acid at amino acid position 33, was achieved by changing the nucleotide sequence coding for hirudin by an overlap PCR reaction. GAC was replaced by TGT in the coding sequence of mutant hirudin. The amplified DNA fragment was cloned in the 2-micron based, high copy number yeast expression vector, pDP 34, as described in [2]. Cells of the matured culture broth were removed by centrifugation and proteins showing anti-thrombin activity (see below) were removed from the supernatant by adsorption on XAD-7 resin. A mixture of active proteins was eluted with ammonium acetate buffer (0.2 M, pH 8.5) and applied to a Q-Sepharose fast-flow ion-exchange column. This column was eluted with ammonium formate buffer (25 mM), using a gradient elution from 0% A (buffer adjusted to pH 5.0 with formic acid) to 85% B (buffer adjusted to pH 3.0 with formic acid and containing 0.5 M sodium chloride) within 50 min. Fractions showing anti-thrombin activity were pooled and lyophilized to dryness (crude Cys^{33}-hirudin).

2.2. *Folding of reduced/denatured Cys^{33}-hirudin*

The crude Cys^{33}-hirudin (4 mg) was reduced for 2 h at 23°C in 0.75 ml of Tris-HCl buffer (0.5 M, pH 8.4) containing 5 M GdmCl and 0.1 M dithiothreitol. The sample was passed through a PD-10 column (Pharmacia) equilibrated in 50 mM sodium bicarbonate buffer, pH 8.3. The reduced/denatured sample was collected in 1.2 ml and was immediately diluted with the same sodium bicarbonate buffer to a final concentration of 1 mg/ml (140 μM). The folding was carried out at 23°C and folding intermediates were trapped in a time-course manner by acidifying aliquots of folding sample with 2 vols. of 4%

trifluoroacetic acid. Wild-type hirudin (HV 1) was reduced/denatured and allowed to refold under identical conditions both in the absence and presence of β-mercaptoethanol (140 μM).

2.3. *HPLC characterization of the folding intermediates*

Acid-trapped folding intermediates were directly analysed by HPLC using the conditions described in the legend to Fig. 1.

2.4. *Characterization of the Cys[33]-hirudin monomer and dimer*

Amino acid composition was determined by the dimethylaminoazobenzene sulfonyl (dansyl) chloride method [15], which allows direct quantitation of the disulfide content. Amino acid sequence was analysed by an Applied Biosystem Inc. 470A protein sequencer. The antithrombin activity of hirudin derivatives was assessed by their ability to inhibit a-thrombin from digesting Chromozym TH (Boehringer Mannheim), using the described protocol [16,17]. Molecular weight of hirudin was determined by the laser desorption ionization mass spectrometry using a home-built instrument [18].

3. RESULTS AND DISCUSSION

3.1. *In vitro folding of the Cys[33]–Cys[33]-hirudin dimer*

Crude Cys[33]-hirudin was shown to be heterogeneous by HPLC. At least 7–8 species were observed (see inset in Fig. 1, top panel). However, analysis by peptide mapping, sequencing and amino acid composition revealed that all these products are made of one species of primary sequence. The crude Cys[33]-hirudin was reduced, carboxymethylated and digested by lysyl endopeptidase and V8 protease, its peptide mapping differs from that of wild-type hirudin by only one peptide, which was isolated and found to contain the sequence, Cys-Gln-CmCys-Val-Thr-Gly-Glu (residues 33–39). The heterogeneity of the crude product thus must arise from improper folding after biosynthesis and secretion or sample destruction during purification.

In order to obtain a pure species of folded Cys[33]-hirudin, we have therefore performed this folding experiment. The process and results turned out to be surprisingly straightforward. Correct folding of reduced/denatured Cys[33]-hirudin took place spontaneously in alkaline buffer alone. Active Cys[33]-hirudin monomer formed first (Fig. 1, top panel) with a first order rate constant of 0.05 ± 0.01 min^{-1} under the experimental conditions described. Oxidation of Cys[33] between two active Cys[33]-hirudin monomers subsequently occurred and the cystine-linked dimer was generated. The folding was highly efficient with a yield of > 85–90%.

The Cys[33]–Cys[33]-hirudin dimer contains no free cysteine, as confirmed by amino acid analysis, and has an anticipated molecular mass of 13,916, as determined by laser desorption mass spectrometry. Both the Cys[33] monomer and the dimer are highly active anti-coagulants and their potencies of thrombin inhibition are nearly indistinguishable (± 3%). At a concentration of 1 nM of hirudin and 1.5 nM of α-thrombin, the dimer exhibits, on an equal weight basis, 82–86% of the inhibitory activity of wild-type hirudin. This suggests that one hirudin dimer binds to two molecules of α-thrombin, which is not unexpected since the thrombin binding sites of hirudin are apparently not impaired as a result of dimerization (Fig. 2).

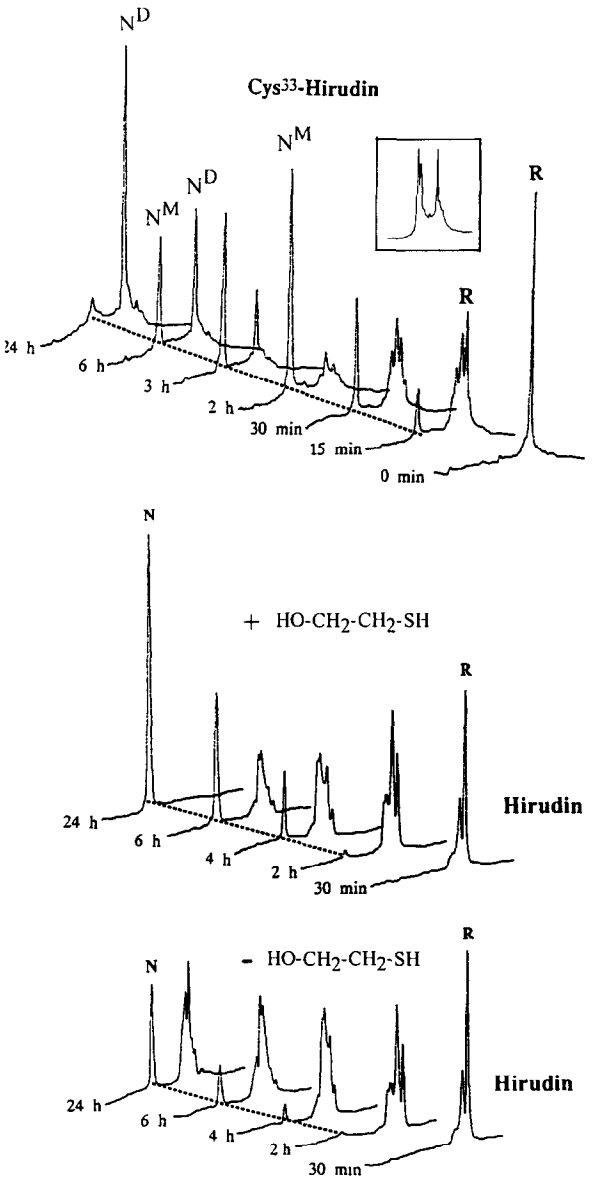

Fig. 1. The process of folding of reduced/denatured hirudins. Cys[33]-hirudin in the buffer alone (top panel). Wild-type hirudin in the buffer containing 140 μM of β-mercaptoethanol (middle panel). Wild-type hirudin in the buffer alone (bottom panel). The buffer was 50 mM $NaHCO_3$ (pH 8.3). The folding intermediates were trapped by acid in a time-course manner and analysed by HPLC using the following conditions. Column was Vydac C_{18} for peptides and proteins, 5 mm; solvent A was water containing 0.1% trifluoroacetic acid; solvent B was acetonitrile/water (9:1, v/v) containing 0.1% trifluoroacetic acid. The gradient was 24–43% solvent B linear in 20 min; peptides were detected at 214 nm. Reduced/denatured (R) Cys[33]-hirudin and wild-type hirudin were eluted at 17.7 min and 18.8 min, respectively. 'N' is native wild-type hirudin (14.1 min). 'N^M' is active Cys[33]-hirudin monomer (13.5 min). 'N^D' is Cys[33]-hirudin dimer. In the absence of β-mercaptoethanol, about 60% of the wild-type hirudin was trapped as scrambled 3-disulfide species (see the 24h sample of bottom panel). Inset, crude Cys[33]-hirudin analysed by the same HPLC conditions.

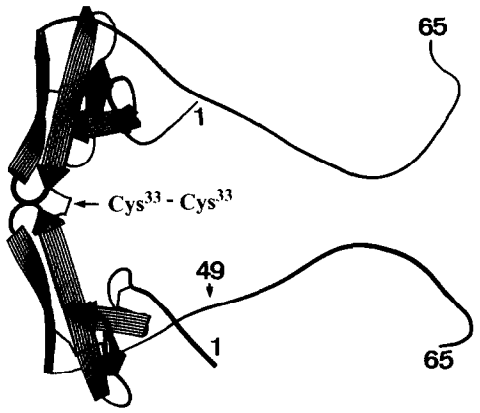

Fig. 2. Proposed structure of the Cys^{33}–Cys^{33}-linked hirudin dimer. Hirudin contains a compact N-terminal domain (residues 1–49) and a disordered C-terminal tail (residues 50–65). The α-thrombin binding site of the N-terminal domain is opposite to the Cys^{33}. The three native disulfides within the monomer are Cys^{6}–Cys^{14}, Cys^{16}–Cys^{28} and Cys^{22}–Cys^{39}.

3.2. *The dimer and monomer are inter-convertible*

Unlike the three intra-disulfide bridges of hirudin, the Cys^{33}–Cys^{33} of the dimer is not stabilized by non-covalent interactions. It may therefore be possible to selectively cleave Cys^{33}–Cys^{33} under mild reducing conditions without disrupting the three disulfides located within the active core domain. Experiments were carried out in order to find out an optimum condition that would allow quantitative conversion of the dimer to the monomer. This can be achieved by using 0.5–2.5 mM of β-mercaptoethanol at room temperature (Fig. 3). At higher concentration of β-mercaptoethanol, the monomer rapidly disintegrates as a consequence of the reduction of Cys^{6}–Cys^{14}, Cys^{16}–Cys^{28} and Cys^{22}–Cys^{39}.

3.3. *The folding mechanism of Cys^{33}-hirudin*

The folding intermediates of Cys^{33}-hirudin were trapped by acidification and analysed by HPLC (Fig. 1, top panel). The results are compared to those obtained from the folding of wild-type hirudin carried out both in the absence and presence of β-mercaptoethanol (140 μM) (Fig. 1, middle and bottom panel).

The folding pathway of hirudin has been recently shown to undergo a two-stage process of packing/consolidation [19]. The course of packing was characterized as unfolded hirudin flowing sequentially and irreversibly through [equilibrated 1-disulfide isomers]→[equilibrated 2-disulfide isomers]→[equilibrated 3-disulfide (scrambled) isomers]. The mechanism of consolidation involved reorganization and refinement of the scrambled species to attain the active structure. The efficiency of packing and consolidation, in our case, can be selectively regulated and conditions can be chosen to allow the completion of hirudin folding within 10 min or up to 10 h. The rate of packing is primarily enhanced by the oxidized glutathione, whereas the process of consolidation requires free thiols (reduced glutathione or β-mercaptoethanol) as catalyst. In the presence of oxidized glutathione (2–10 mM) alone, the flow of packing completes within 2 min and consolidation become the major rate limiting step. As a consequence, scrambled hirudins become the only detectable folding intermediates under these conditions. In the absence of both oxidized glutathione and supplementing free thiols, about 60% (this number is dependent upon the concentration of protein employed for folding) of the starting material was trapped as scrambled 3-disulfide species which were unable to convert to the native hirudin (Fig. 1, bottom panel). This problem could be overcome by including catalytic amounts of β-mercaptoethanol (or other free thiols such as reduced glutathione) in the folding sample (Fig. 1, middle panel).

Based on the HPLC patterns, we conclude that Cys^{33}-hirudin most likely folds via similar mechanisms. Due to the presence of an extra cysteine (Cys^{33}), active Cys^{33}-hirudin monomer formed quantitatively without additional thiol reagents. It is nonetheless interesting to observe (top and middle panels of Fig. 1) that the folding of active Cys^{33}-hirudin monomer is at least 12-fold more efficient than that of wild-type hirudin in the presence of 140 μM β-mercaptoethanol (concentration identical to that of the extra Cys^{33}). These results suggest that

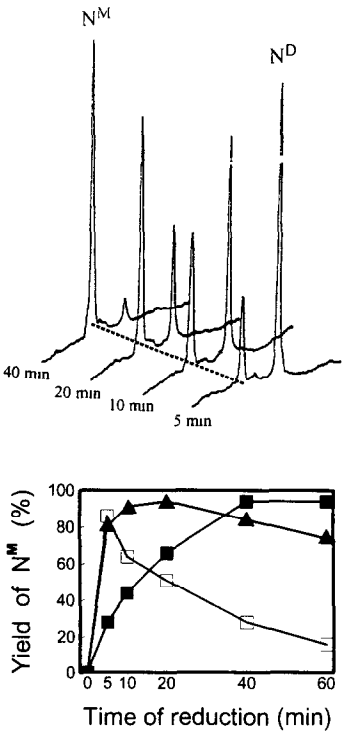

Fig. 3. Coversion of Cys^{33}-hirudin dimer to the monomer under mild reducing conditions. The dimer was dissolved in the folding buffer containing 0.5 mM (■), 2.5 mM (▲) or 10 mM (□) of β-mercaptoethanol. Final concentration of the dimer was 7 μM. Reduction was carried out at 23°C. Partially reduced samples were trapped by acid in a time-course manner and directly analysed by HPLC using the same conditions described in Fig. 1. Chromatograms of samples reduced by 0.5 mM of β-mercaptoethanol are also presented.

Cys^{33} must provide a highly localized concentration of thiols which facilitate the disulfide equilibrium (shuffling) to reach the active structure. Thus, introducing an extra cysteine should, in principle, enhance the efficiency of the folding of cysteine-containing proteins. However, whether or not the introduced cysteine will eventually engage in the dimerization may well depend upon its position in the native structure of the protein. For instance, we speculate that if the cysteine substitution of hirudin was located within the core domain (e.g. Ser^{19} to Cys) instead of the surface loop (Cys^{33}), the efficiency of hirudin folding might be enhanced in the same manner, but the formation of the dimer should not be expected. Similar results of enhancement were observed recently by Weissman and Kim [20]. BPTI derivatives, attached by the Pro region or an extended C-terminal sequence which contain one extra cysteine, refold more efficiently than BPTI itself. These data together point out an unique route of influencing the efficiency of folding of disulfide containing proteins, as well as a simple way of creating disulfide-linked protein dimers.

In conclusion, an efficient method for the large-scale production of hirudin dimer is described. It remains to be seen through pharmacokinetical and clinical analysis whether the hirudin dimer will have the advantage of an increased half-life.

Acknowledgements: We thank R. Voellmy, M. Kamke and F. Leenders for assistance during expression and isolation of the crude products, and Dr. J. Priestle for preparation of Fig. 1.

REFERENCES

[1] Markwardt, F. (1970) Methods Enzymol. 19, 924–932.
[2] Meyhack, B., Hinnen, A. and Heim, J. (1989) in: Genetics and Molecular Biology of Industrial Microorganisms (Hershberger, C.L., Queener, S.W. and Hegemann, G. eds.) pp. 311–321, American Society for Microbiology, Washington, DC.
[3] Markwardt, F. (1991) Thromb. Haemostas. 66, 141–152.
[4] Maerki, W. and Wallis, R.B. (1990) Thromb. Haemostas. 64, 344–348.
[5] Chang, J.-Y. (1983) FEBS Lett. 164, 307–313.
[6] Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. and Fenton II, J.W. (1990) Science 249, 277–280.
[7] Gruetter, M., Priestle, J.P., Rahaul, J., Grossenbacher, H., Bode, W., Hofsteenge, J. and Stone, S.R. (1990) EMBO J. 9, 2361–2365.
[8] Chang, J.-Y. (1990) J. Biol. Chem. 265, 22159–22166.
[9] Mao, S.J.T., Yates, M.T., Owen, T.J. and Krstenansky, J.L. (1988) Biochemistry 27, 8170–8173.
[10] Maraganore, J.M., Chao, B., Joseph, M.L., Jablonski, J. and Ramachandran, K.L. (1989) J. Biol. Chem. 264, 8692–8698.
[11] Haruyama, H. and Wuetrich, K. (1989) Biochemistry 28, 4301–4312.
[12] Folker, P.J.M., Clore, G.M., Driscoll, P.C., Dodt, J., Koehler, S. and Gronenborn, A.M. (1989) Biochemistry 28, 2601–2617.
[13] Steiner, V. (1988) Diplom. Thesis, University of Basel, Switzerland.
[14] Dodt, J., Muller, H.-P., Seemuller, U. and Chang, J.-Y. (1984) FEBS Lett. 165, 180–184.
[15] Chang, J.-Y. and Knecht, K. (1991) Anal. Biochem. 197, 52–58.
[16] Stone, S.R. and Hofsteenge, J. (1986) Biochemistry 25, 4622–4628.
[17] Chatrenet, B. and Chang, J.-Y. (1992) J. Biol. Chem. 267, 3038–3043.
[18] Boernsen, K.O., Schaer, M. and Widmer, M. (1990) Chimia 44, 412–416.
[19] Chatrenet, B. and Chang, J.-Y. (1993) J. Biol. Chem. 268, 20988–20996.
[20] Weissman, J.S. and Kim, P.S. (1992) Cell 71, 841–851.