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Stability and folding studies of two homologous proteins, human stefins A and B.

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Dedicated to the memory of Professor Savo Lapanje

Abstract: In a shorter Introduction we describe the usual experimental means of how to study protein stability and folding. Our work on stability of human stefins A and B, determined by chemical, heat and pH denaturation, is described next. We explain the folding mechanism of human stefin B (as determined thus far) and compare it to homologous human stefin A, in particular, the dependence of folding rates on the concentration of GuHCl. pH denaturation of human stefin B (E.Žerovnik et al., Eur. J. Biochem. 1997, 245, 364-372) and its folding to the native state are described in more detail.

Introduction

Denaturation [1,2] studies allow the measurement of some basic thermodynamic properties of proteins, which also can be calculated from coordinates of the 3D structures [3]. The mechanism of folding is more difficult to determine as there may be many routes and many intermediate states leading to the native state. The major drive to the native state stems from thermodynamics even though, there are cases where kinetic barriers route proteins into trapped intermediates or meta-stable native states [4]. It is now apparent that 3-state folding with a sequentially placed intermediate on the route from unfolded to the native state is no more an exception [5-7]. The "old" and "new"

views of protein folding [8,9] differ mainly in the way they describe this complex reaction. The old view stresses common conformations - states, whereas the new view points to each separate conformation [9].

Experimentalists collect data on thermodynamics and kinetics of protein folding. One usually starts by determining the stability from heat, pH and chemical denaturation. If multiple spectroscopic probes give coincident transitions this means that there are no populated intermediates. 2-state transition is also revealed by identity of Van't Hoff and calorimetric enthalpies which are obtained by differential scanning calorimetry (DSC).

To determine the kinetics of folding and unfolding, lots of data have to be collected under different experimental conditions. The changes should be studied by different spectroscopic probes which monitor different structural aspects of the process. Rate constants should be determined as a function of denaturant concentration, temperature, pH and selected mutations. Even though the kinetic data can be fit to plausible models these still need to be confirmed. One useful experiment to distinguish between intermediate and native states is measuring unfolding after various times of refolding [10].

New biophysical techniques [11] permit results of high quality and time resolution. For example, advances have been made in multidimensional NMR, where the difficult task of investigating the denatured and partially folded states is achievable [12, 13]. To see which h-bonded structures of protein become protected during folding, quenching of hydrogen to deuterium exchange is followed by NMR or MS measurements. The changes in compactness during folding are getting measured using small angle X-ray scattering [14].

Human stefins A and B were chosen for studies of protein stability and folding due to their relative simplicity : small M_r and no disulphide bonds. Both posess a number of prolines which are in the trans conformation. Sequence similarity of stefins A and B amounts to 56 % and they fold in a very similar 3D conformations (less than 1 Å

deviation of the backbone C-atoms). Despite these similarities they exhibit marked differencies in stability and mechanism of folding. We have determined stability and kinetics of folding using several spectroscopic probes, and have measured the kinetics by fluorescence as a function of temperature, pH and GuHCl concentration. In order to determine the rate-limiting step, the folding was started from various denatured states.

Denaturation and folding studies of human stefins A and B

The two proteins were first isolated from human sources [15-17]. Later, many homologues were identified in the animal kingdom [18-20] and in plants [21]. In Table 1, some representative aminoacid sequences are aligned. Human stefins A and B are members of the cystatin superfamily of cysteine proteinase inhibitors [21,22]. They have been cloned and expressed in E.coli [23,24] and the yield of expression subsequently improved [25]. The 3 D structures of each are determined by X-ray crystallography (human stefin B in complex with papain [26] and by solution NMR (human stefin A [27, 28]).

Initial study was done on folding of human stefin A [29]. GuHCl denaturation [29] , monitored by near UV CD, far UV CD and tyrosine fluorescence, with a midpoint at 2.8 ± 0.1 M GuHCl (25^{o}C, pH 8), showed a reversible, two state unfolding transition. The kinetics of slow folding was measured (10^{o}C, pH 8) by the same three probes. In 2.0 M GuHCl, the kinetics were rather slow (k = 0.002 s^{-1} by all three probes). The amplitudes of the observable phases were 90±10 % by tyrosine fluorescence and near UV CD, but only 20% by the far UV CD. This can be interpreted as kinetically 2-state folding from an initial intermediate possessing 80% of the signal in the far UV CD. "Double jump" experiments were performed and have shown that the slow changes in tyrosine fluorescence reach their complete amplitude only after longer times of unfolding. Except for proline isomerization this might be due to slow unfolding of the core.

In subsequent years, we have focused on recombinant human stefin B. Its denaturation by heat, acid and GuHCl, monitored by fluorescence, near UV and far UV CD [30], were found noncoincident which was explained by the presence of a molten globule - type [31-34] of intermediate [30, 35]. In contrast to stefin A which is thermally extremely stable, the behavior of stefin B is quite different. Differential scanning calorimetry (DSC) was used to obtain thermodynamic data from the thermal unfolding of both proteins [36] as well as some cystatins [37]. The reasons for the big difference in thermal stability between stefins A and B were sought using structural thermodynamics [38]. Reversible DSC scans of stefin A could be analysed in terms of a 2-state transition whereas stefin B unfolded in an irreversible transition (at pH 8 and 6.5, 0.13 M salt). The asimetry of the DSC peaks was characteristic of unfolding of a dimer. At pH 5, the transition of stefin B became reversible and permitted thermodynamic analysis [36].

Table 1

Sequence alignment of some representative stefins; the N-terminal extension is omitted from pig leukocyte CPI (5 residues) [20]. The bold letters stress identity to human stefin B, underlined are residues which are the same to human stefin A. Recombinant human stefin B differs in the third residue (Ser3) from the wild type Cys3.

res No	10	20	30	40	50
human r. stef.B	**MMSGAPSATQ**	**PATAETQHIA**	**DQVRSQLEEK**	**YNKKFPVFKA**	**VSFKSQVVAG**
bovine stefin B[44]	**MMCG**GT**SATQ**	**PATAETQ**A**IA**	**D**K**V**K**SQLEEK**	E**NKKFPVFKA**	LE**FKSQ**L**VAG**
human stefin A	**M**IPDDL**S**EAK	**PATPEIQEIV**	**DKVKPQLEEK**	T**N**ETYGKLE**A**	**V**QY**KTQVVAG**
bovine stefin A[19]	**M**IP**G**GLTEAK	**PATIEIQEIA**	NM**V**KP**QLEEK**	T**N**ETYEE**FTA**	IEY**KSQVVAG**
pig leuko. CPI[20]	**M**LA**G**GLTEPR	**PATPEIQEIA**	N**K**V**K**P**QLEEK**	T**NK**TYEK**F**E**A**	IIYR**SQVVAG**

res No	60	70	80	90	
human r.stef.B	**TNYFIKVHVG**	**DEDFVHLRVF**	**QSLPHENKPL**	**TLSNYQTNKA**	**KHDELTYF**
bovine stefin B[44]	K**NYFIKV**Q**V**D	ED**DFVHI**R**VF**	E**SLPHENKP**V	A**L**TS**YQTNK**G	R**HDELTYF**
human stefin A	**TNY**Y**IKV**RA**G**	**D**NKYM**HLK****VF**	K**SLP**GQ**N**ED**L**	V**L**TG**YQ**VD**K**N	**KDELTGF**
bovine stefin A[19]	I**NY**Y**IK**IQT**G**	**D**NRY**I**H**I**K**VF**	K**SLP**QQSHS**L**	I**L**TG**YQ**VD**K**T	**KDELA**G**F**
pig leuko. CPI[20]	**TNY**Y**IKVHVG**	G**N**NY**V**H**IRVF**	**QSLPH**Q**EDPL**	K**L**IG**YQ**VD**K**T	**KDELTGF**

pH denaturation of human stefin B was studied in more detail by spectroscopic and DSC techniques [39]. The pH titration was performed at "low" salt (buffers described in [39], 0.033 M NaCl) or at "high" salt (the same buffers [39], 0.42 M NaCl). Near and far UV CD spectra were recorded as a function of pH. Some representative far UV CD spectra are shown in Fig1. The pH denaturation was recorded at 200, 208, 222 and 277 nm, and

showed two transitions, at pH 4.1 and 5.7. At pH 5.7 only minor changes at 200, 208 and 222 nm are observable (due to minor changes in secondary structure) which is supported by recent NMR experiments on N^{15} labeled stefin B. At pH 4.1, rather large and cooperative changes occur at all wavelengths. The final, acid intermediate (denatured) state I_1 (below pH 3.3) has some properties of a "less structured" molten globule [33], in particular, in binding ANS. Adding salt to I_1 produces a transition to a "more structured" molten globule I_2. All CD and fluorescence data were acquired at 18^{o}C as the population of intermediate states changes with temperature. Instead of

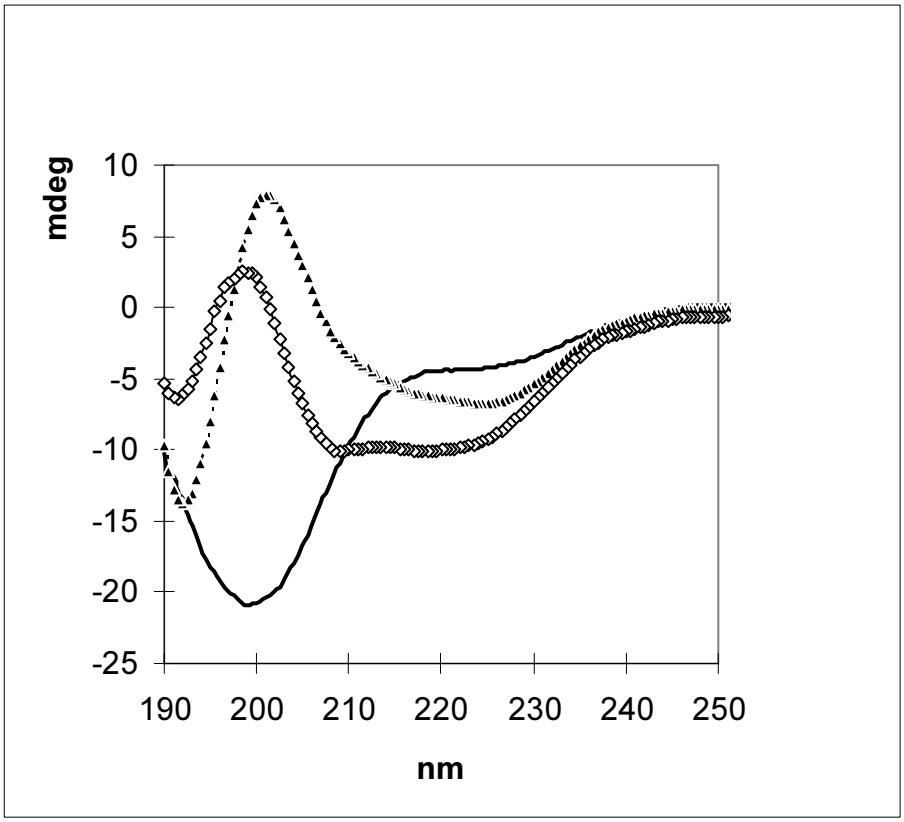

Figure 1. Far UV CD spectra of human stefin B in various denatured and intermediate states: N (0.01 M phosphate buffer pH 6.5), I_2 (0.01 glycine 0.25 M sulphate, pH 3.3), I_1 (0.01 M glycine , pH 3.3); from top to the bottom at 200 nm.

studying pH denaturation at several temperatures, we have heated samples at various pH values. DSC data [39] have shown that thermal denaturation deviates from 2-state with decreasing pH which was explained by a rather low enthalpy of unfolding of the acid intermediate state I_1. As this state is populated to 100 % only below pH 3.3 where

enthalpy could not be measured due to the low melting temperature, no firm conclusion on the enthalpy of unfolding of state I_1 could be made. In contrast, the second, more structured acid intermediate I_2 demonstrates a measurable enthalpy of unfolding.

^{1}D NMR spectra [39] of the acid induced intermediates of human stefin B have been measured and have confirmed that I_2 posesses some tertiary structure (high field methyl resonances) in contrast to I_1. It is hoped that by using N^{15} and C^{13} labelled samples and acquiring 2D and 3D NMR spectra much more detail would be obtained also of the other two acid intermediate states I_1 and I_2. (R.J., experiments underway). The compactness of the acid intermediate states and their oligomeric nature was probed by SEC [40].

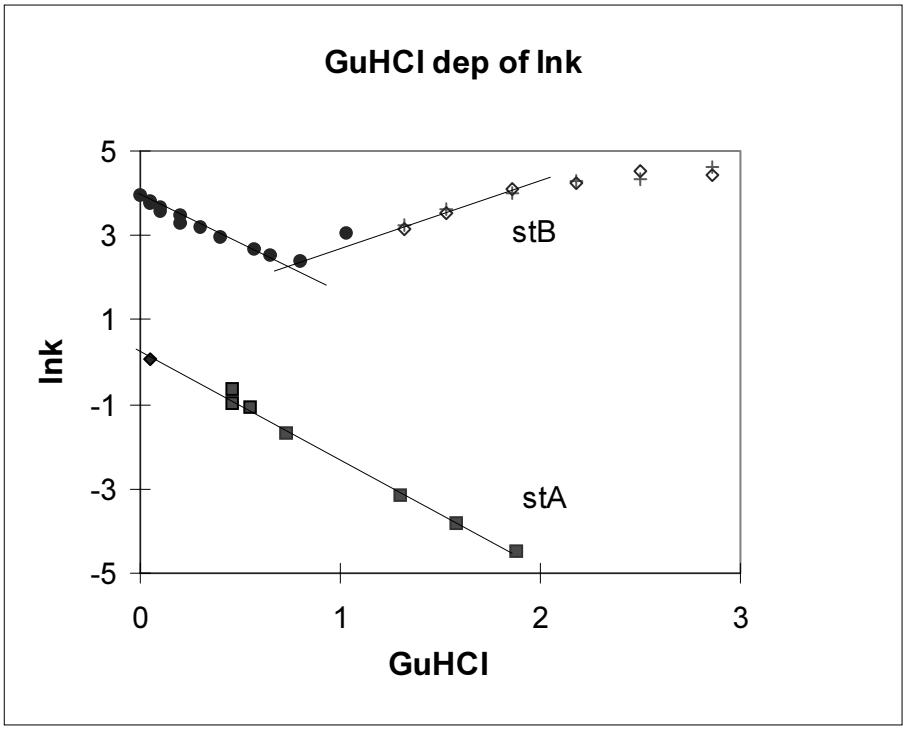

Figure 2. Logarithm of the rate constants against GuHCl molarity. Folding of human stefin B was started from the acid intermediate state at pH 2.7. This was found the same as when started from the GuHCl denatured state at 3.45 M GuHCl. (not shown). Unfolding was started from the native state at pH 6. Folding of human stefin A was started from the GuHCl denatured state (4.4 M GuHCl). The initial values at zero GuHCl have been obtained by folding the acid denatured state to zero GuHCl at otherwise the same conditions (pH 6, 20^{o}C).

The folding mechanism of stefin B has been studied [41,42] and compared to that of stefin A. We also have studied the influence of pH and TFE on the folding reaction[41]. Using several spectroscopic probes (CD in the near, CD in the far, tyrosine and ANS fluorescence - Table 2), it was found that the kinetics of folding of human stefin B differ from those of stefin A. Not only is the major phase of folding 40-times faster (at zero GuHCl, - Fig.2), this protein demonstrates an additional slow phase. The slow phase of 25 % amplitude (Table 2) could be ascribed to proline isomerization but pH dependence of the amplitude and double jump experiments do not prove it univocally. An intermediate seems a more likely hypothesis. In the very initial stages of folding, a "burst" phase of the signal at 230 nm was observed [41]. Its amplitude was 60% ± 5% of the total change.

In a second paper [42], folding was started from different initial states : GuHCl unfolded, TFE denatured, acid denatured and acid intermediate state. The folding rates were found to be the same, so that the rate limiting step must also be the same. In Fig.3, folding from acid denatured state A_D is shown as monitored by ANS fluorescence. ANS binding on folding of stefin B and many other proteins is very informative. The initial increase can be explained as compactization [32,34] which is supported by X-ray scattering [43]. The decrease of ANS fluorescence occurs along with appearance of the native-like states (Fig.3 B,C).

To conclude : Many more experiments still have to be done to be able to understand the folding of these two homologous proteins. We hope to study their mutants and to follow dimerization reactions which seem connected to stefin B folding. In cooperation with other laboratories it will be possible to determine the rate of compactization (time-resolved X-ray scattering [43] and details of structure formation (real time NMR [45]).

Table 2

Comparison of the folding kinetics of human stefins A and B as measured by the near and far UV CD, tyrosine and ANS fluorescence. Data are taken from [41]. The folding of stefin B was started by 6-times dilution from 3.45 M GuHCl and of stefin A from 4.4 M GuHCl, both, to pH 6. The measurements were done at 20 ^{o}C. Rate constants are in s^{-1} and amplitudes as % of the observed change. "Burst phase" amplitudes in the far UV CD are 60% and 45 % of the total change, respectively, for stefins B and A. No amplitude is given for ANS fluorescence, only the sign.

human stefin B folding

probe	k_1'	k_1/A_m	k_2'	k_2/Am
Tyr fl.		14.2/77%		0.05/23%
ANS fl.	86(+)	12.8(-)	0.34(+)	0.022(-)
CD far		10.5/76%		0.045/24%
CD near		10.8/75%		0.039/25%

human stefin A folding

probe	k / Am
Tyr fl.	0.18 / 100%
ANS fl.	no change
CD far	0.18 / 100%
CD near	0.16 / 100%

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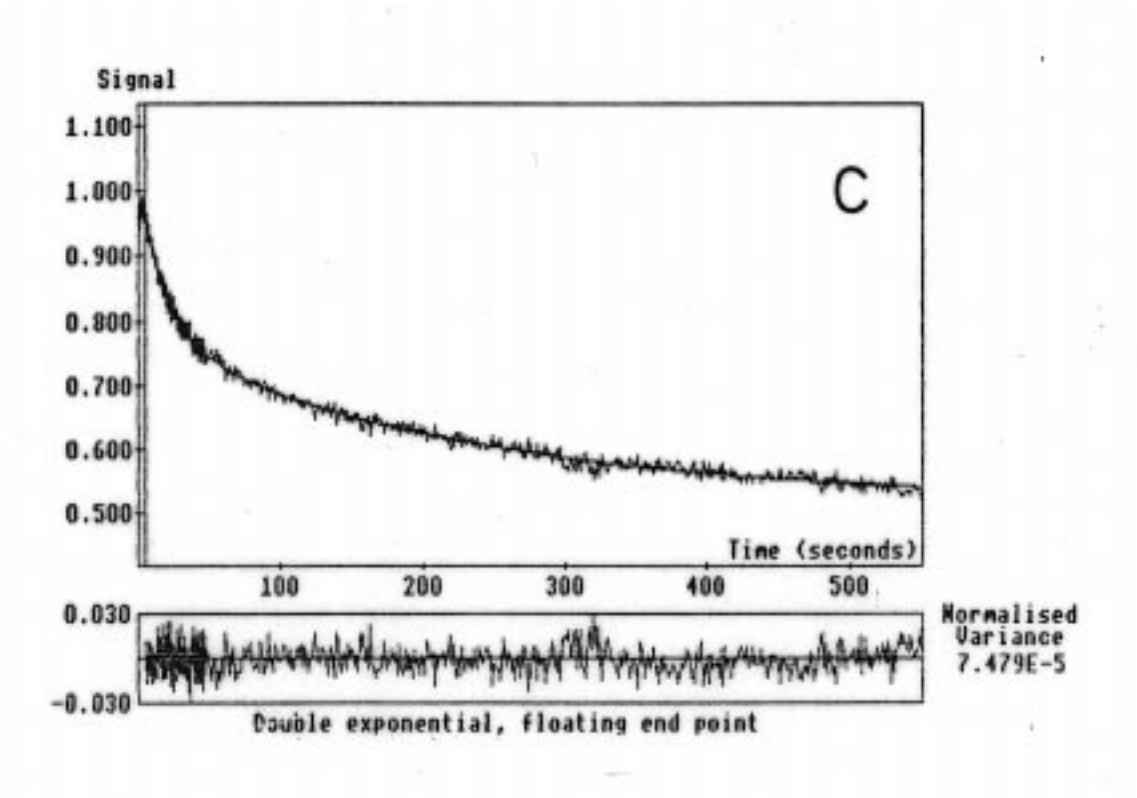

Figure 3. Kinetics of folding of human stefin B recorded by ANS fluorescence. The folding was started from state A_D at pH 1.8 ± 0.2 (dialysis against water / HCl pH 1.8) and proceeded into 0.02 M phosphate buffer. After 6-fold dilution the final pH was 6.0 ± 0.1.
A) the initial ANS fluorescence increase - folding to an intermediate ($k'' = 533$, $k' = 92\ s^{-1}$)
B, C) folding to the native state is tri-phasic ($k_1 = 11\ s^{-1}$, $k_2 = 0.053\ s^{-1}$ and $k_3 = 0.004\ s^{-1}$)

Povzetek : Najprej je podan kratek uvod v eksperimentalni del študija stabilnosti in foldinga proteinov. Nato opisujemo naše delo s človeškima stefinoma A in B. Kinetiko foldinga stefina B smo merili z več probami in jo primerjali s kinetiko foldinga stefina A. Merili smo tudi odvisnost hitrosti od koncentracije GuHCl, pH-ja in temperature. pH denaturacija človeškega stefina B (E.Žerovnik et al., Eur.J.Biochem. 1997, 245, 364 - 372) in folding do nativnega stanja iz kislo denaturiranega, sta opisana nekoliko bolj natančno.

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