# Kinetic stabilisation of a modular protein by domain interactions

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**Abstract Protein S, a two-domain spore coat protein from *Myxococcus xanthus*, is structurally related to eye-lens βγ-crystallins. No natural monomeric one-domain member of this protein superfamily is known. To determine the stability of the single domains and to explain the ubiquitous domain duplication, the isolated domains of protein S were constructed. The N-domain is thermodynamically more stable than the C-domain. In intact protein S, domain interactions lead to an apparent decrease in stability of the N-terminal domain, whereas the C-terminal domain is stabilised. In contrast, unfolding kinetics of both domains are decreased 100-fold due to interactions in the complete molecule.**



*Key words:* βγ-Crystallin; Domain folding; Differential scanning calorimetry; Kinetic stabilization; Two-domain protein; Domain interaction

## 1. Introduction

Polypeptide chains beyond a critical length of 50–100 amino acid residues tend to form domains as structurally independent entities [1,2]. The βγ-crystallin superfamily is a most illuminating example to illustrate this hierarchical principle of protein structure and domain duplication [3], especially because it allows the oligomerisation of crystallins and their homologs to be explained by 'molecular domain swapping' [2,4].

Protein S, the spore coat forming protein of the soil bacterium *Myxococcus xanthus*, is structurally related to the superfamily of βγ-eye-lens crystallins [5–7]. It shares not only their Greek-key topology and their domain size but also their high intrinsic stability [2]. However, in contrast to the crystallins, it polymerises in a $Ca^{2+}$-dependent manner to form a durable cuticula, thus protecting the syncytium upon desiccation or other stress conditions [8].

The domains of βγ-crystallins exhibit a surprisingly high structural homology. However, under suitable conditions, independent domain folding has been observed. Examples to illustrate this feature are bovine γB-crystallin [9] and protein S [10]. The well-separated equilibrium transitions of their domains suggest significant differences in their intrinsic stabilities. In the case of γB-crystallin, differences in the thermodynamic stability and the 3D structure of the domains were determined using carefully selected domain boundaries [11,12].

In order to examine the stability of the isolated N- and C-terminal domains (NPS and CPS) of protein S, we cloned the domains and purified the recombinant proteins after expression in *Escherichia coli*. Since CPS turned out to be significantly less stable than NPS, it was supplemented with a $His_6$ tag in order to speed up the purification and to enhance the yield. As shown by absorbance, fluorescence emission and circular dichroism spectroscopy, the isolated domains exhibit characteristics of native protein S. They are monomeric entities without any tendency to combine to the 'nicked' two-domain parent molecule; both are stabilised by $Ca^{2+}$ binding (manuscript in preparation).

## 2. Materials and methods

### 2.1. Cloning and purification

Molecular cloning procedures were based on Sambrook et al. [13]. The plasmid pETPS used for cytoplasmic expression of protein S has been described elsewhere [10]. The plasmids for expression of the isolated domains, pETNPS and pETCPS, were constructed by cloning the corresponding genes into the plasmid pET11a [14] via the *Nde*I and *Bam*HI restriction sites. For CPS, a sequence encoding a $His_6$ tag was added at the C-terminus. Recombinant protein S and its N-terminal domain were expressed and purified as described for protein S [10]. After expression in *E. coli* strain BL21(DE3) [15], the C-terminal domain was purified using a Ni-NTA resin and gel filtration.

### 2.2. Differential scanning calorimetry (DSC)

DSC studies were performed using a Nano-DSC CSC 5100 calorimeter (Calorimetry Sciences Corp., Provo, UT, USA) at a constant heating rate of 1 K/min. All experiments were carried out in 20 mM sodium cacodylate, 3 mM $CaCl_2$, pH 7.0. Samples were dialysed against a 1000-fold volume of buffer. Buffers were filtrated and degassed by evacuation; protein solutions were centrifuged and degassed before the concentration was determined by absorption spectroscopy. Solvent baselines and samples were measured under identical conditions. To examine the reversibility of folding, the protein was cooled down after the first unfolding transition, and heated up a second time. Data were analysed with the deconvolution software CpCalc 2.1 of the CSC 5100 [16].

### 2.3. Kinetic experiments

Folding and unfolding kinetics were monitored using absorption spectroscopy (286 nm, path length 1 cm) for protein S and NPS, and far-UV CD spectroscopy (222 nm, path length 0.1 cm) for CPS: Cary 1/3 Varian spectrophotometer and AVIV 62D spectropolarimeter. All experiments were carried out in 25 mM MOPS, 3 mM $CaCl_2$, pH 7.0 at 20°C. Unfolding of native protein and refolding of unfolded protein (CPS in 5 M urea, protein S in 7.5 M urea and NPS in 7 M urea) were initiated by dilution with solutions containing varying concentrations of urea to give the appropriate folding conditions and final protein concentrations of 0.3 mg/ml for CPS and 0.6 mg/ml for protein S and NPS. The individual kinetics were analysed as mono-exponential or double-exponential functions for NPS, CPS and protein S, respectively. The dependence of the rate constants $k$ on the urea concentration were analysed assuming a two-state mechanism of folding and a linear dependence of the logarithms of the microscopic rate constants of unfolding $k_u$ and of refolding $k_f$ on urea concentration. In the figures, the fitted curves resulting from this analysis are given as solid lines.

Stopped-flow kinetic experiments for the fast refolding of the N-terminal domain were performed using a SX-18MV sequential mixing stopped-flow spectrometer (Applied Photophysics, Leatherhead, UK). The path length of the observation chamber was 1 cm, and the ki-

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*Abbreviations:* NPS, N-terminal domain of protein S; CPS, C-terminal domain of protein S


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netics were monitored at 20°C by the change in absorbance at 286 nm. To initiate refolding, solutions of 5 mg/ml unfolded NPS in 7 M urea were diluted 11-fold with urea solutions of varying concentrations to give final concentrations of 0.6–4 M. Kinetics were measured six times under identical conditions, averaged and analysed as mono-exponential functions using the software provided by Applied Photophysics.

## 3. Results and discussion

Thermal analysis by DSC and denaturation/renaturation kinetics were applied in order to quantify the stability of NPS and CPS.

Fig. 1 and Table 1 illustrate the reversible thermal denaturation of $Ca^{2+}$-liganded protein S and its isolated domains at pH 7.0. While the DSC profiles of NPS and CPS are monophasic and show two-state behaviour ($N \leftrightarrow U$), the unfolding of protein S can only be fitted assuming a three-state model ($N \leftrightarrow I \leftrightarrow U$), with two nearly coinciding equilibrium transitions at 65 and 66°C. The corresponding enthalpy changes, $\Delta H = 26$ and 19 kJ/mol, reflect the nearly cooperative unfolding of the two domains, with the first transition attributable to the melting of CPS (including the loss of domain interactions), the second transition to the unfolding of NPS. Comparing the DSC profiles of the isolated domains, similar values for the change in molar heat capacity, $\Delta C_p$, and for $\Delta H$ are observed, as one would expect for proteins with similar size and fold [17]. On the other hand, there is a striking difference of 20 degrees between the melting points of the two domains, reflecting the significantly higher thermodynamic stability of the N-terminal domain compared to its C-terminal counterpart. In addition, in the complete two-domain protein, NPS apparently loses stability by its interaction with CPS: its $T_M$ is shifted from 70 to 66°C (Table 1).

Summarising the DSC data, protein S exhibits a close similarity to other modular proteins which were previously shown to gain stability by mutual interactions of their domains, to the extent that in certain cases separate domains were found to require their counterparts as 'chaperones' [18].

A second alternative to gain insight into the significance of domains in connection with the stability and folding of modular proteins is the analysis of denaturation/renaturation *kinetics*. Fig. 2 shows the dependence of the apparent rate constants $k$ of unfolding and folding on the denaturant concentration. For the isolated domains only mono-exponential kinetics were observed, as for other all-β proteins with fewer than 90 amino acid residues [19]. In contrast, in the case of the complete two-domain protein S, only the unfolding kinetics can be fitted with mono-exponential functions, where-

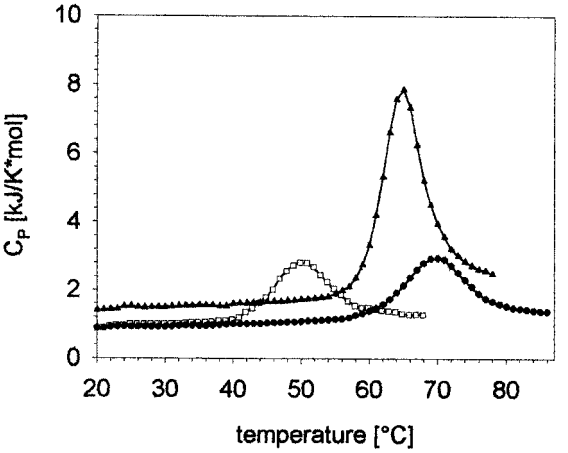


Fig. 1. Comparison of the DSC melting profiles of protein S and its isolated domains. (□) CPS (0.82 mg/ml); (●) NPS (1.78 mg/ml); (▲) protein S (1.53 mg/ml). Buffer: 20 mM sodium cacodylate, 3 mM $CaCl_2$, pH 7.0, heating rate 1 K/min.

as refolding shows biphasic kinetics up to a urea concentration of 3.5 M. In addition, the 'roll-over' at low denaturant concentrations clearly indicates the occurrence of folding intermediates at $c_{urea} < 2$ M [20], thus rendering the extrapolation of ln $k$ to zero denaturant concentration impossible [21]. Only for NPS and CPS, thermodynamic parameters are accessible by extrapolation to $c_{urea} = 0$ (Table 1). The results are in agreement with data obtained from the urea-induced equilibrium transitions (manuscript in preparation). However, comparing the kinetics of the separate domains at $c_{urea} \rightarrow 0$ with those of intact protein S, a most surprising result is obtained: both the highly stable NPS and the unstable CPS unfold at the same rate ($k_u \approx 9 \times 10^{-5}$ $s^{-1}$; cf. Fig. 2A,C), whereas in intact protein S the unfolding of the cooperative unit of the interacting domains is slowed down 100-fold ($k_u \approx 9 \times 10^{-7}$ $s^{-1}$, cf. Fig. 2B). This leads us to suggest that intact protein S is stabilised kinetically due to a high energy barrier between the native and unfolded states. As a consequence, its folding rate is expected to be slowed down [22]. A comparison of the folding rates of NPS ($k_f = 21$ $s^{-1}$) and CPS ($k_f = 0.13$ $s^{-1}$) with the respective slow and fast kinetic phases calculated for protein S at low urea concentration is in accordance with this idea ($k_f \approx 2.5 \times 10^{-3}$ and $1.4 \times 10^{-2}$ $s^{-1}$). The deviation from two-state behaviour in the case of protein S clearly indicates a complex folding mechanism with intermediates highly populated under native-like solvent conditions. In this connection,

Table 1
Thermodynamic and kinetic parameters for protein S and its isolated domains NPS and CPS in the presence of 3 mM $Ca^{2+}$

| | Protein S (18.6 kDa, 172 aa) | NPS (9.4 kDa, 88 aa) | CPS (10.2 kDa, 91 aa) |
|---|---|---|---|
| Thermodynamic parameters | | | |
| $T_M$ (°C) | 65/66 | 70 | 50 |
| $\Delta H$ (kJ/mol) | 26/19 | 20 | 18 |
| Reversibility (%) | 91 | 95 | 98 |
| Kinetic parameters | | | |
| $c_{1/2urea}$ (M) | 4.8 | 5.2 | 2.5 |
| $\Delta G_{(20°C,pH7.0)}$ (kJ/mol) | – | 30 | 20 |
| $k_f$ ($s^{-1}$) | – | 21 | 0.13 |
| $k_u$ ($s^{-1}$) | $9.0 \times 10^{-7}$ | $8.5 \times 10^{-5}$ | $9.6 \times 10^{-5}$ |

$T_M$, melting point; $\Delta H$, enthalpy change; reversibility, calculated using $(\Delta H_{2ndheating}/\Delta H_{1stheating}) \times 100$; $c_{1/2urea}$, concentration of urea at which 50% of the molecules are unfolded; $\Delta G_{(20°C,pH7.0)}$, Gibbs free energy of folding at 20°C and pH 7.0, calculated according to $\Delta G = -RT \ln K_D$, with $K_D = k_u/k_f$; $k_f$, microscopic rate constant of folding in the absence of urea; $k_u$, microscopic rate constant of unfolding in the absence of urea.

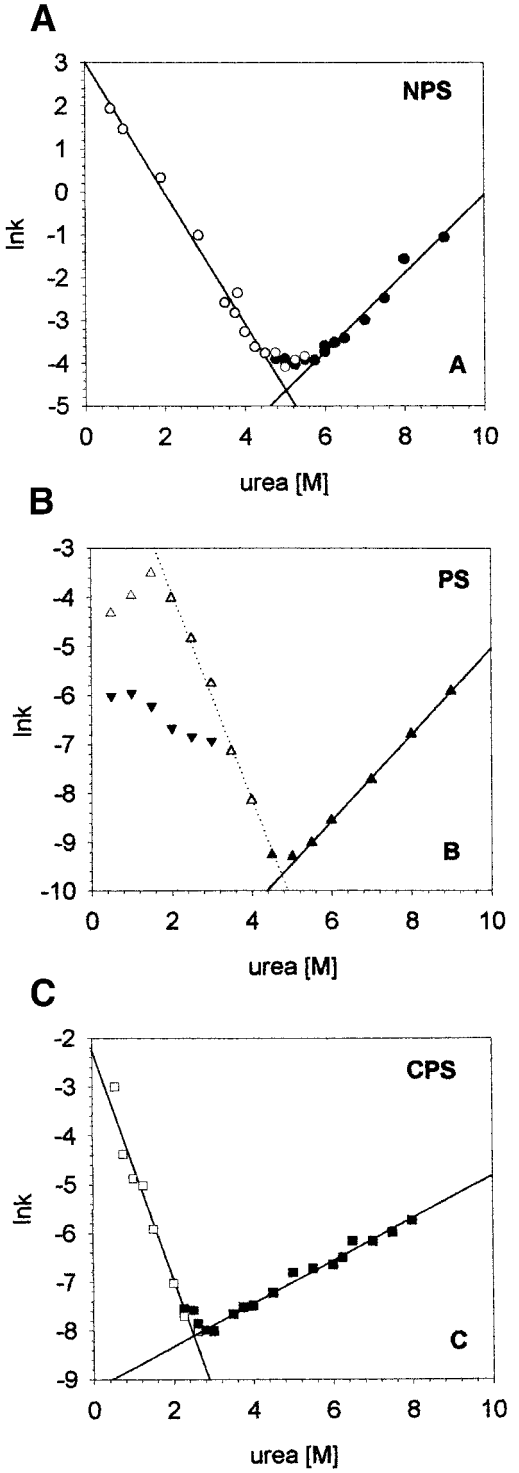


Fig. 2. Reaction rate profiles of the folding and unfolding kinetics of the isolated domains NPS and CPS, and protein S at 20°C, 25 mM Mops, 3 mM $CaCl_2$, pH 7.0. A: NPS (0.6 mg/ml). Method of detection: absorbance at 286 nm. (●) unfolding, (○) refolding. B: Protein S (0.6 mg/ml). Method of detection: absorbance at 286 nm. (▲) unfolding, (△) refolding fast phase, (▼) refolding slow phase. C: CPS (0.3 mg/ml). Method of detection: far-UV CD spectroscopy at 222 nm. (■) unfolding, (□) refolding; solid lines represent the fit of the data according to two-state unfolding.

a significant loss of amplitude at an early stage of the folding kinetics clearly indicates rapid folding of the N-terminal domain within the dead-time of the experiment. The slow phase, with a decrease in rate compared to isolated CPS, may be attributed to the C-terminal domain, the rate-limiting step to the pairing of the domains. Based on this mechanism, the kinetic stabilisation of protein S seems to reside mainly in specific domain interactions. According to the 3D structure of the protein [6,7] the C-terminal tyrosine 121 (involved in two hydrogen bonds with N-terminal backbone amide groups in the interface) might be a good candidate for these specific interactions. Preliminary mutation studies involving this position indicate a destabilisation of protein S and independent domain folding (unpublished data).

## 4. Conclusions

In conclusion, despite their structural similarity, the two domains of protein S exhibit drastic differences in their folding kinetics and intrinsic stabilities. The latter are equalised in the intact protein, leading to an apparent monophasic equilibrium unfolding transition of the intact two-domain protein. In spite of the slight upward shift of the melting temperature observed for NPS compared with the N-terminal domain within intact protein S, both domains gain stability in the intact protein because of the kinetic barrier which slows down the unfolding transition. Comparing these results with the properties of the structurally related γB-crystallin, significant differences become obvious: in γB-crystallin, the N-terminal domain is not affected in its stability, whereas the stability of the isolated C-terminal domain is strongly decreased [11]; hydrophobic domain interactions contribute significantly to the intrinsic stability [23].

The question why domains with identical folds differ in their stability properties remains open. It may be correlated with different functions, in the case of protein S possibly connected with the stress-induced folding and polymerisation.

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