Engineering a two-helix bundle protein for folding studies

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The SAP domain from the Saccharomyces cerevisiae THO1 protein contains a hydrophobic core and just two α-helices. It could provide a system for studying protein folding that bridges the gap between studies on isolated helices and those on larger protein domains. We have engineered the SAP domain for protein folding studies by inserting a tryptophan residue into the hydrophobic core (L31W) and solved its structure. The helical regions had a backbone root mean-squared deviation of 0.9 Å from those of wild type. The mutation L31W destabilised wild type by 0.8 ± 0.1 kcal mol⁻¹. The mutant folded in a reversible, apparent two-state manner with a microscopic folding rate constant of around 3700 s⁻¹ and is suitable for extended studies of folding.

Keywords: α-helix/model protein/protein structure/two-helix bundle/two state folding mechanism

Introduction

There are extensive studies of the folding kinetics of very small peptides to analyse isolated individual helices (Thompson et al., 1997; Huang et al., 2001; Du et al., 2007) and of three- to five-helix bundles to study protein domains with hydrophobic cores (Munson et al., 1994; Huang and Oas, 1995; Burton et al., 1998; Ferguson et al., 1999, 2005; Kubelka et al., 2003; Mayor et al., 2003; Wang et al., 2003; Jemth et al., 2004; Sato et al., 2004; Horng et al., 2005; Korzhnev et al., 2007; Yang et al., 2008; Wensley et al., 2009). Here we have established a system to bridge the gap between these studies by analysing the folding events of a two-helix bundle, the SAP domain from the THO1 protein from Saccharomyces cerevisiae (PDB accession numbers 1h1j and 2wqg; Fig. 1).

The SAP domain is the only domain identified in the 218 amino acid THO1 protein, where it is found at the N-terminus. While the C-terminal portion of the protein plays a role in the control of transcription elongation by RNA polymerase II (Jimeno et al., 2002, 2006), the role of the N-terminal SAP domain remains unclear. Comparison with homologous proteins (Aravind and Koonin, 2000) suggests that it may be involved in targeting the C-terminal domain to actively transcribed DNA. The SAP domain has a hydrophobic core formed from conserved leucine residues (L13, 17, 31 and 35) and is stable in solution at millimolar concentrations. We introduced a tryptophan fluorophore into the core of the domain to report on kinetic transitions, and conducted equilibrium and kinetic studies to determine its suitability for extended protein folding studies.

Results

Fluorophore probe and solution structure

The THO1 SAP domain does not possess an intrinsic tryptophan probe suitable for monitoring the folding kinetics, so we engineered tryptophan residues into various core locations of SAP in order to obtain a suitable, minimally perturbing fluorescence probe. The most suitable mutant to act as a pseudo-wild type was SAP L31W. It expressed in milligram quantities in Escherichia coli and was stable in solution at millimolar concentrations. The equilibrium unfolding of both wild-type and pseudo-wild-type proteins was monitored by CD at 222 nm. Both displayed reversible apparent two-state transitions (Fig. 2). L31W was destabilised by 0.8 ± 0.1 kcal mol⁻¹ relative to the wild-type construct at its midpoint (Table I).

The solution structure of L31W was determined de novo using NMR spectroscopy (Table II). The domain consists of two α-helices (13 and 14 residues, respectively) connected by a structured loop of five residues. The N-terminal tail, eight residues long, was structured from residue 3 onwards and contained a single turn of α-helix, whereas the C-terminal tail of 11 residues appeared disordered throughout (Fig. 1). The structure of L31W was almost identical to that of the wild type (backbone root mean-squared deviation (RMSD) of 0.9 Å over helices), with minor rearrangement of the loop packing to accommodate the larger residue (Fig. 1C and D), backbone RMSD rising to 1.3 Å over helices and loop). This structure was consistent with the alignment tensor calculated from measured residual dipolar couplings (RDCs) of the amide bond (correlation coefficient of 0.93 for ordered regions where S² > 0.8, 0.90 over helices and loop, 0.93 over helices only).

 Backbone dynamics of the amide vector was measured to probe the mobility of L31W (Fig. 3A–C. Formal analysis of the measured rates using the program Tensor2 (http://eva.ibs.fr/ext/labos/LRMN/softs/) to calculate the order parameter,
confirmed that the helices and most of the connecting loop are ordered \( S^2 > 0.8 \), Fig. 3D). Overall, the similarity between the solution structure of SAP L31W and that of the wild-type protein showed that the mutant would be a suitable pseudo-wild-type protein for folding studies.

**Tuning the stability of L31W**

We determined whether variations in the ionic strength or pH modulated the stability of SAP L31W (Fig. 4A and B, filled circles) in order to identify the optimal conditions for in vitro studies of protein folding reactions (Ferguson and Fersht, 2008). Ideally, such studies should be performed under conditions where the protein stability is independent of small changes in pH, so that changes in pH with temperature (arising from non-zero ionisation enthalpies for buffers) or batch-to-batch variation in reagents have negligible effects.

L31W had near constant stability between pH 5.5 and 9.5 in thermal denaturation studies. However, SAP L31W had a marked increase in stability with increasing [NaCl] from 150 mM to 1.5 M, at pH 6.0 most likely resulting from an increasing screening of surface charge clusters. We tuned the stability of L31W by varying the [NaCl] to optimise the definition of baselines within the experimentally accessible window in order to aid curve fitting. We used urea for chemical denaturation studies as it does not affect the ionic strength.

Increasing the ionic strength from 150 to 500 mM with NaCl stabilised the construct by 0.6 kcal mol\(^{-1}\) and when combined with a reduction in temperature from 298 to 283 K resulted in an overall increase in the L31W stability of 1.1 kcal mol\(^{-1}\). Urea denaturation of L31W at 283 K in this optimised buffer gave data with well-defined baselines (Fig. 5 for example curve). Five repeat denaturations, each monitored at 100 single wavelengths (see Materials and methods for exact averaging procedure), yielded an average midpoint, \( D_{50} \), of 4.8 ± 0.1 M urea and an \( m \)-value, \( \partial \Delta G_{D-N}/\partial [\text{urea}] \), of 700 ± 60 cal mol\(^{-1}\) M\(^{-1}\) (standard error from replicates).

The apparent \( \Delta C_p \) of denaturation (the difference in the heat capacity between native and denatured states) for L31W was determined from a plot of \( \Delta H \) versus \( T_m \) determined by measuring thermal denaturation as a function of solvent pH (Privalov, 1979). Since \( \Delta C_p \) is a constant parameter in the fitting of thermal transitions, we used an iterative process of fitting transitions and plotting \( \Delta H \) versus \( T_m \) until there was no further change (Fig. 4C). The value so determined was 520 ± 40 cal mol\(^{-1}\) K\(^{-1}\) (errors using a ‘jack-knifing’ procedure of deleting each value in turn and refitting the data in its absence). This value was similar to that estimated using the method of Myers et al. (1995) assuming 14 cal mol\(^{-1}\) per residue but using only the number of ordered residues (calculated \( \Delta C_p \) of 532 cal mol\(^{-1}\) K\(^{-1}\)).
Temperature-jump measurements of the folding kinetics of L31W in different concentrations of urea yielded linear arms in chevron plots of logarithm of observed rate constant versus concentration of chemical denaturant (Fig. 6). The curves were deconvolved in three ways: the most widely used ‘unconstrained’ fit where all parameters are allowed to float freely (Jackson and Fersht, 1991), a ‘constrained’ fit where the reference urea concentration is the denaturation midpoint (determined from equilibrium measurements and further repeat with crosses (L31W only)).

Table I. Thermal denaturation of wild-type and L31W SAP

|          | Wild type | L31W pseudo-wild type |
|----------|-----------|-----------------------|
| $T_m$ (K) | 327.1 ± 0.1 | 320.2 ± 0.3           |
| $\Delta H$ at $T_m$ (kcal mol$^{-1}$) | 40.5 ± 0.6 | 35.0 ± 1.2 |
| $\Delta G_{237-K}$ at 327 K (kcal mol$^{-1}$) | N/A | 0.49 ± 0.0 |

$^a$Thermal denaturation was carried out in 50 mM MES, pH 6.0, total ionic strength of 150 mM (wild type) and 500 mM (L31W) made up with NaCl. Initial scan shown in filled circles, repeat scan with open circles and further repeat with crosses (L31W only).

The chemically denatured state of THO1 L31W appeared random-coil-like

The C$_\alpha$ secondary shifts (the difference between the measured chemical shift and the expected random coil value) of L31W were measured in 8 M urea to provide an indication of structure in non-native states (Williamson, 1990; Wishart et al., 1991; Mittag and Forman-Kay, 2007). Regions of positive secondary shifts measured for the native state correlated well with the determined helices and are destroyed in the presence of urea (Fig. 7). Thus, the C$_\alpha$ chemical shifts for all residues in the urea-denatured state are close to those expected in a random coil and did not provide any evidence of residual structure upon denaturation.

Discussion

L31W as a paradigm two-helix protein

SAP L31W is a suitable system for studying the folding of two-helix bundle proteins. The SAP domain was monomeric at up to millimolar concentrations as determined by NMR measurements of both wild-type and pseudo-wild-type proteins (C.A. Dodson, T.J. Rutherford and J.O.B. Jacobsen, unpublished results). SAP L31W denatured reversibly without aggregation and the equilibrium unfolding transition fitted well within the experimentally accessible window (0–10 M urea, 275–371 K) and multiple repeats of thermal denaturation of both wild-type and pseudo-wild-type SAP
domains superimposed (Fig. 2). There was also a wide pH
range (Fig. 4A, filled circles) in which the folding and
unfolding reactions of SAP L31W were well defined using
equilibrium and kinetic biophysical methods.

The tryptophan residue inserted into the core of wild-type
SAP domain at position 31 minimally perturbed its structure
(Fig. 1). The fluorescence reported on the conformational
state of the protein. The presence of the tryptophan did not
alter its major biophysical properties (the stability of both
wild-type and pseudo-wild-type proteins showed the same
pH and ionic strength dependencies—Fig. 4A and B, filled
and open circles, respectively).

The chemical denaturation curves of L31W were well
defined with good baselines for native and denatured regions
under all conditions, apart from the native state at low ionic
strength. Addition of NaCl tuned the stability of L31W so
that under optimised conditions both native and denatured
baselines were well defined (Fig. 5). In this way, the ionic
strength dependence of the stability of L31W can be
exploited, making future Φ-value analysis possible. This is
fortunate, since the best mutations for Φ-value analysis are
often destabilising and thus have truncated pre-transition
baselines compared with the wild-type protein. The acquisi-
tion of accurate stability measurements for marginally
stable small proteins is complicated as over-truncation

Fig. 3. Backbone dynamics and order in L31W. (A) Longitudinal, (B)
transverse relaxation times and (C) heteronuclear NOE enhancement for
L31W. Errors are set to 10% of measured value, which is consistent with the
error from five repeat measurements of R2 relaxation rate constants.
(D) Order parameter for L31W. The order parameter reports on the
proportion of the measured relaxation, which can be attributed to global
motion. A value of $S^2 = 1$ indicates that relaxation is due to global motion
alone; if $S^2 = 0$, relaxation is due to only internal motion. Errors shown are
fitting errors. The anomalously low value for L27 in all four panels is an
artefact due to overlap of the HSQC peak with that of D4.

Fig. 4. Biophysical characterisation of the SAP domain. (A) pH and (B)
ionic strength dependency of stability for L31W (filled circles) and
wild-type SAP (open circles). pH buffers were 50 mM buffer salts, total
ionic strength of 500 mM (L31W) and 150 mM (wild type) made up with
NaCl, ionic strength was made up to stated value with NaCl in 50 mM MES,
pH 6.0. Error bars show a fixed error of 0.6 K (three standard deviations
(SD) of an estimated error of 0.2 K). (C) Determination of $\Delta C_p$ for L31W
using acid (filled circles) and base (open circles) perturbation of L31W. The
intrinsic error of the measurement of $\Delta H$ was greater than the error
introduced by non-zero total ionisations, so points from basic perturbation of
SAP were included in the overall fit in order to increase the precision of
$\Delta C_p$ determination. The slope of the fitted line is 520 cal mol$^{-1}$ K$^{-1}$ and the
error bars are set to 1 kcal mol$^{-1}$.
impinges on the precision of the thermodynamic parameters determined by curve fitting.

**L31W displayed simple folding kinetics**

Kinetic transients for L31W were monophasic and the observed rate constants displayed a classic chevron plot with linear arms (Fig. 6). Measurement of L31W kinetics on longer timescales or using laser T-jump apparatus (capable of measuring nano-second kinetics) gave a trace with single exponential decays and no evidence of phases faster or slower than those measured here.

Both the kinetic midpoint, $D_{50}^{\text{kin}}$ (the [urea] at $k_{50}$ where $k_f = k_u$), and the total $m$-value, $m_{\text{tot}} (= m_f + m_u)$, determined from unconstrained fits were in agreement with the equilibrium measurements (Table III compared with $D_{50}$ and $m_{\text{eq}}$ from urea denaturation—parameters in main text above), although the propagated error in $D_{50}^{\text{kin}}$ was large, which is inherent in studies on proteins with low values of $m_u$.

**L31W is a paradigm for two-helix bundle protein folding studies**

L31W is one of the simplest naturally occurring protein domains possible and can be considered either as two helices oriented by a connecting loop or a small hydrophobic core being protected from solvent by essentially only a single layer of solvating residues. This minimal composition bridges the divide between studies of isolated elements of secondary structure, which focus on local interactions, and larger globular domains where long-range forces may dominate. L31W is a suitable model protein for extensive studies on folding including $\Phi$-value analysis.

**Materials and methods**

**Reagents**

Wild-type SAP domain (GSADYSSLTVQLKDLLTKRN LSVGGLKNEVLQRLIKDDEESKGESEVSPQ) and L31W mutant were over-expressed in *E. coli* C41 cells as His-tagged, C-terminal fusion proteins. Unlabelled recombinant proteins were expressed in 2XTY rich media, whereas isotopically enriched samples for NMR experiments were expressed in a modified K-Mops medium (Sattler et al., 1999) using 0.1% (w/v) [15 N]ammonium chloride and 0.4% (w/v) [U-13C]glucose (Cambridge Isotopes Laboratories, Andover, MA) as the sole N and C sources. The wild-type plasmid (a derivative of pRSETa) was the kind gift of Dr Mark Bycroft (MRC Centre for Protein Engineering) and the L31W mutant was generated from this by the Stratagene Quikchange mutagenesis procedure. Fused proteins were isolated from clarified cell lysate by Ni-charged IMAC resin (GE Healthcare BioSciences, Sweden). SAP domain was cleaved from fusion protein with a protease cleavage at 37°C for 3 h using ~100 units of bovine plasma thrombin (Sigma, UK). After cleavage, the SAP domains were purified to
Table III. Kinetic data for folding and unfolding of SAP L31W

|                         | Fit constrained using equilibrium  $K_{eq}$ | Fit constrained using equilibrium  $D_{30}$ | Unconstrained fit* |
|-------------------------|------------------------------------------|--------------------------------------------|--------------------|
| $m_i$ (cal mol$^{-1}$ M$^{-1}$) | 460 ± 10                                  | 410 ± 10                                   | 430 ± 20           |
| $m_o$ (cal mol$^{-1}$ M$^{-1}$) | 180 ± 10                                  | 120 ± 40                                   | 280 ± 110          |
| $k_{on}^C$ (s$^{-1}$)     | 3710 ± 120                                | 2680 ± 360                                 | 3530 ± 150         |
| $k_{on}^D$ (s$^{-1}$)     | 15 ± 1                                    | 26 ± 9                                     | 4 ± 5              |
| $k_{off}^C$ (s$^{-1}$)    | 80 ± 10                                   | 70 ± 0                                     | 90 ± 10            |
| $m_{tot}$ (cal mol$^{-1}$ M$^{-1}$) | 630 ± 10                                 | 580 ± 40                                   | 720 ± 110          |
| $D_{30}$ (M)              | 4.9 ± 0.1                                 | 4.8 ± 0.1$^d$                             | 5.4 ± 1.4          |
| $\Delta G_{283}^{CH}_{N}$ (kcal mol$^{-1}$) | 3.1 ± 0.0                                | 2.8 ± 0.2                                 | 3.8 ± 0.7          |

*aNon-italicised quantities are parameters derived directly from the curve-fitting expression. Italicised quantities are calculated from these. Errors are fitting errors (or errors propagated from these).

$b k_{on}$ is the microscopic rate constant at the midpoint, i.e. at $k_{30}$, $k_t = k_u (= k_{50})$.

$c m_{tot} = m_i + m_o$.

$d D_{30}$ for constrained fit is determined by equilibrium denaturation.

NMR spectroscopy

NMR spectra were acquired at 283 K on Bruker DRX 500 or AVANCE 800 spectrometers equipped with 5 mm inverse detection triple resonance probes. $^1$H–$^1$N heteronuclear single quantum coherence (HSQC), $^1$H–$^1$C HSQC, HNCA, CBCA(CO)NH, HNCACB, CBBC(CO)NH, HN(CA)CO and HNCO spectra were acquired for backbone assignments (Neri et al., 1989; Johnson et al., 1996). These spectra were augmented by H(CCCO)NH homonuclear 2D TOCSY (55 ms MLEV-17 spin-lock in 7.1 kHz B$_1$ field), 2D NOESY (120 ms $r_m$) and DQF-COSY spectra to obtain side-chain assignments and distance restraints. Methyl resonances of valine and leucine residues were assigned stereospecifically from the sign of correlations in a constant-time $^1$H–$^1$C-HSQC obtained for fractionally (10%) $^{13}$C-labelled THO1 (Delaglio et al., 1995). Chemical shifts were referenced to external 3-trimethylsilylpropanesulphonic acid. All data were processed in NMRPipe (Wuthrich, 1986; Bax, 1994), and analysed in the program Sparky (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco, CA). Assignment of backbone and side-chain resonances, followed by assignment of cross-peaks in the 2D $^1$H–$^1$H NOESY spectrum, proceeded by standard methods (McDonald and Thornton, 1994). Hydrogen-bond donor amide groups were identified from the slowly exchanging peaks in H–D exchange experiments. $^1$H–$^{15}$N HSQC spectra were recorded after dissolving lyophilised protein-buffered deuterium oxide. Cognate hydrogen bond acceptors were identified by manual inspection of structures and the program hbplus (Cornilescu et al., 1999).

The input to iterative rounds of structure calculations comprised, in order of inclusion, Nuclear Overhauses Effect (NOE) intensity-derived distance restraints in four categories (corresponding to inter-proton distance constraints of 1.8–2.7, 1.8–3.3, 1.8–5.0 and 1.8–7.0 Å), side-chain torsion angle restraints obtained from stereo-specific assignments, $\pi$ and $\varphi$ angle restraints obtained from secondary shifts of backbone resonances using the program TALOS (Stein et al., 1997) and fixed hydrogen bond distance constraints.

Fig. 7. $C_a$ secondary shifts for L31W in 0 M (red) and 8 M (blue) urea. Shading indicates boundaries of helices determined from final structure. Apparent large values in the denatured state can be rationalised as the C-terminal residue (Q51) and the residue immediately preceding a proline (S49), which is expected to show a negative offset of 1–2 ppm.

homogeneity using ion exchange (6 mL Resource S column; GE Healthcare BiSciences, Sweden) and reverse phase chromatography (0–50% (v/v) acetonitrile gradient, 0.1% (v/v) trifluoroacetic acid using a C4 column). Proteins were lyophilised to remove volatile solvents. The identity and mass of purified proteins were confirmed by SDS–PAGE and matrix-assisted laser desorption ionisation mass spectrometry. All reagents were purchased from Sigma (Sigma-Aldrich Corp., St Louis, MO), BDH or Fisher Scientific and most were AnalaR grade or higher, with the exception of ultrapure urea that was purchased from MP Biomers.

Buffers

All experiments were measured in 50 mM buffer salts with the ionic strength adjusted to the stated value using NaCl. Water with resistivity of 18 M$\Omega$ cm was used for dilution of all reagents, and urea concentrations were accurately determined using a refractometer. Buffers used were 50 mM Na formate (pH 3.5–4), Na acetate (pH 4–5.5), MES (pH 6–6.5), Na phosphate (pH 7–8.5) and ethanolamine (pH 9–10.5) with ionic strengths corrected to 500 mM using NaCl. For studies on the effects of ionic strength we used 50 mM MES pH 6.0 with ionic strength adjusted to the stated value with NaCl. The buffer used for the final studies under optimised conditions was 50 mM MES pH 6.0, with the ionic strength adjusted to 500 mM with NaCl, at 283 K where appropriate.
Structures were calculated using a standard torsion angle-simulated annealing protocol (Brunger et al., 1998) in the program CNS (Nicholson and Scholtz, 1996).

$^{1}$D$_{NT}$ RDCs were measured by TROSY and IPAP-HSQC sequences in micelles formed from 5% (w/v) aqueous C$_{12}$E$_{5}$ polyethylene glycol/octanol (Ottiger et al., 1998; Ruckert and Otting, 2000). $^{15}$N T$_{1}$ values were obtained from 13 standard inversion-recovery HSQC spectra, varying the relaxation delays between 4 ms and 1.6 s. $^{15}$N T$_{2}$ values were obtained from 12 HSQC spectra modified with a CPMG sequence of delays between 4 ms and 1.6 s. 15NT 2 values were obtained using the Sparky software package.

Equilibrium thermal denaturation
Far-UV CD spectroscopy or differential scanning calorimetry (DSC) was used to measure the thermal denaturation of wild-type and pseudo-wild-type proteins. For all measurements except the ionic strength dependence of wild-type SAP domain, the change in differential absorbance at 222 nm was measured using a Jasco J-720 or J-815 spectropolarimeter (Jasco Inc., Easton, MD) with temperature controlled by a Peltier unit. Samples generally contained 50 $\mu$M protein and were measured in a 1 mm pathlength cuvette. Samples were heated from 280 to 360 K using a scan rate of 60 K/h and data were fit to a two-state transition (Pace, 1986; Jackson and Fersht, 1991). The change in the heat capacity ($\Delta C_p$) was used as a constrained parameter ($\Delta C_p = 520$ cal mol$^{-1}$ K$^{-1}$). The thermal stability of wild-type SAP domain on varying ionic strength was measured using a VP-Capillary DSC (MicroCal/G E Healthcare). Samples containing 300 $\mu$M protein were heated at a scan rate of 120 K/h between 2 and 115°C and the resulting transitions fitted using the MicroCal Origin software supplied with the instrument.

Equilibrium chemical denaturation
Equilibrium chemical denaturation was monitored by fluorescence emission between 300 and 400 nm using either a PTI QuantaMaster fluorimeter (West Sussex, UK) or a 320 nm cut-off filter on an Aviv 215SF spectrometer. The sample block temperature was controlled using a Peltier unit and a thermocouple used to ensure that the final sample temperature in the cuvette was correct. Excitation was at 280 nm. Solutions of varying concentrations of urea were used for equilibrium denaturation. For curves measured under optimised conditions, all 100 single-wavelength measurements were independently fitted to two-state transitions to determine both the m-value ($\partial \Delta G_{D_{20}} / \partial [\text{urea}]$) and denaturation midpoint ($D_{20}$). Those with fitting errors greater than $\sim 10\%$ were discarded and the remainder averaged, the averaged weighting according to the goodness of fit (fitting error). Results from fitting in this manner were very similar to those from globally fitting all data to a single m-value and $D_{20}$ and also to fits of the wavelengths showing greatest amplitude over the denaturation.

Kinetic measurements
We measured relaxation kinetics on the microsecond to millisecond timescale using T-jump fluorescence spectroscopy.

Temperature jumps of 3–5 K were used to induce the resistive heating on a modified Hi-Tech PTJ-64 (Hi-Tech Ltd., Sailsbury, UK) capacitor-discharge, T-jump apparatus. Filtered solutions were degassed, with stirring, for $\sim 10$ min before kinetic measurements. We acquired and averaged between 8 and 256 traces at each concentration of denaturant measured in order to obtain similar signal-to-noise for reactions with different relaxation amplitudes. Data corresponding to the instrumental rise-time were removed prior to curve fitting and the transients were well described by single-exponential functions under all conditions.

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