Folding and Association of the Human Cell Cycle Regulatory Proteins ckshs1 and ckshs2†

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ABSTRACT: The two human proteins ckshs1 and ckshs2 are each 79 amino acids in length and consist of a four-stranded β-sheet capped at one end by two α-helices. They are members of the cks family of essential cell cycle regulatory proteins that can adopt two native states, a monomer and a domain-swapped dimer formed by exchange of a C-terminal β-strand. ckshs1 and ckshs2 both have marginal thermodynamic stability (the free energies of unfolding at 25 °C are 3.0 and 2.5 kcal/mol, respectively) and low kinetic stability (the rates of unfolding in water are approximately 1 s^{-1}). Refolding of their denatured states to the monomeric forms of the proteins is slowed by transient oligomerization that is likely to occur via domain swapping. The folding behavior of ckshs1 and ckshs2 is markedly different from that of suc1, the cks protein from *Schizosaccharomyces pombe*, but the domain swapping propensities are similar. The greater thermodynamic and kinetic stability of suc1 and the population of a folding intermediate are most likely a consequence of its larger size (113 residues). The similarity in the domain swapping propensities, despite the contrast in other biophysical properties, may be attributable to the common double-proline motif in the hinge loop that connects the swapped domain to the rest of the protein. The motif was shown previously for suc1 to control the equilibrium between the monomer and the domain-swapped dimer. Finally, according to our model, the kinetic barrier separating the monomer and the domain-swapped dimer arises because the protein must unfold for β-strand exchange to occur. Consistent with this, interconversion between the two states is much faster in the human proteins than it is for suc1, reflecting the faster unfolding rates of the former.

The cks[1] (cyclin-dependent kinase subunit) family of proteins are essential for regulation of the eukaryotic cell cycle. Most evidence has pointed to a mitotic role of the cks proteins, and their known function is to bind to and regulate the activity of the major mitotic cyclin-dependent protein kinase (cdk) (*1*, *2*). It is thought that the cks proteins target the cdk to specific substrates by simultaneously binding to the cdk and to a partially phoshorylated protein. Recent studies have indicated a new, cdk-independent function in the G1−S transition, by directing the SCF-mediated ubiquitinylation ligase of $p27^{Kip1}$ (*3*, *4*).

Dimer and hexamer forms have been observed for different members of the cks family, in addition to the monomer (*5*−*8*). The oligomer forms result from domain swapping (*9*−*11*) that occurs via exchange of an internal β-strand, β4, between two monomers to form a dimer pair. The monomer and oligomer structures are superimposable with the exception of the β-turn between strand 3 and strand 4 that acts as a molecular hinge and opens into an extended conformation in the dimer. There are two homologues in humans, known as ckshs1 and ckshs2, and their sequences are 81% identical (*12*). Crystal structures of the ckshs1 monomer (Figure 1A) and the ckshs2 hexamer have been determined (*5*, *7*). The hexamer form is a trimer of strand-exchanged dimers that results in an overall ring structure (Figure 1B). The conserved structure within the family comprises a four-stranded antiparallel β-sheet and two short α-helices. The *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* proteins have, in addition, two large insertions of a long α-helix at the N-terminus and a large loop between the two other α-helices (*6*). The result is a more extensive hydrophobic core in the two larger proteins.

The aim of our studies of the cks proteins is to analyze the sequence determinants of the thermodynamics and kinetic mechanism of domain swapping in vitro. In recent years, more examples of domain-swapped structures have appeared in the literature, and there is some evidence suggesting that domain swapping is a mechanism for association into oligomeric diseased states, such as amyloids (*13*−*15*). However, there is as yet very little information about how domain swapping occurs and whether it is confined to a small number of sequences and/or structures (*15*−*17*). In the absence of such mechanistic detail, the structures of domain-swapped proteins and their relationship to disease states remain purely phenomenological. We have studied the folding and oligomerization behavior of suc1, the cks protein from *S. pombe* (*18*−*20*). The human cks are much smaller than suc1, and size is the only significant difference between

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[1] Abbreviations: CD, circular dichroism; cdk, cyclin-dependent kinase; cks, cyclin-dependent kinase subunit.

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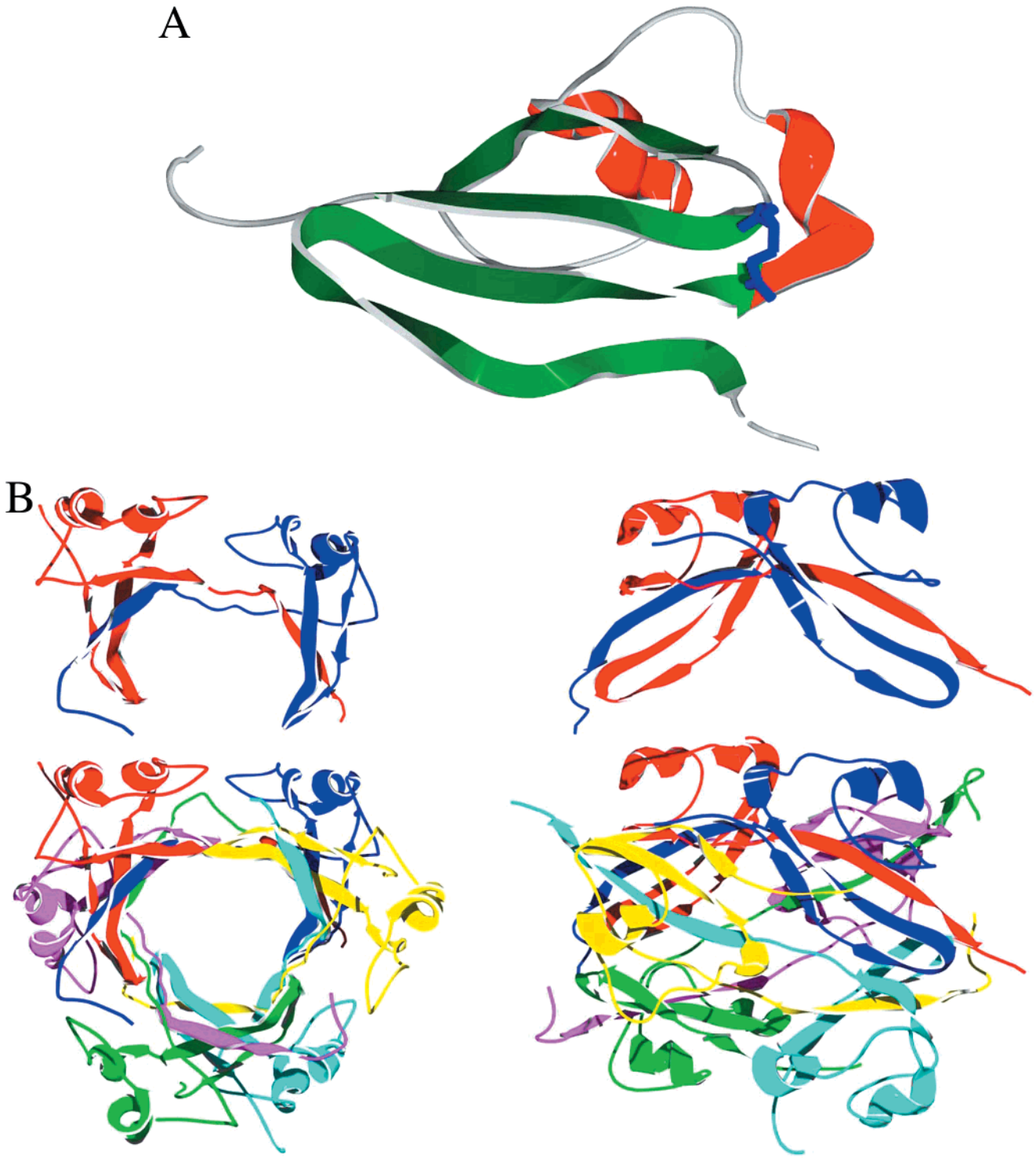

FIGURE 1: Schematic representation of (A) the ckshs1 monomer structure with the side chain of residue Glu63 shown and (B) the ckshs2 hexamer structure.

these proteins, the level of sequence identity being very high within each of the common secondary structural elements. How does this difference affect the folding and oligomerization behavior? Further, what constraints on the folding behavior are imposed by the high level of sequence identity that is most likely a consequence of the conserved function in this family of proteins? Finally, the functional significance of domain swapping in these proteins has yet to be established. Characterization of the phenomenon in vitro should provide clues for helping to answer this question.

EXPERIMENTAL PROCEDURES

Highly pure urea was obtained from Rose Chemicals Ltd. All other chemicals were obtained from Sigma or BDH.

Cloning and Mutagenesis. cDNA for ckshs1 and cDNA for ckshs2 were synthesized from HeLa cell mRNA using a first-strand cDNA synthesis kit (Pharmacia). These were then amplified by PCR and cloned into the *Bam*HI and *Eco*RI sites of the pRSET A vector (Novagen) for expression of a six-His tag N-terminal fusion protein. Correct clones were

identified by sequencing (Oswel, University of Southampton, Southampton, U.K.). Site-directed mutagenesis was performed using the Quick Change kit (Stratagene), and mutant plasmids were identified by sequencing.

Protein Expression and Purification. C41(DE3) cells were transformed with the plasmid, and a small number of colonies were picked from the plate into flasks of 1 L of 2TY medium containing 0.5 mg/mL ampicillin. The cells were grown at 37 °C to an OD_{600} of ~0.6 and then induced with 0.5 mM IPTG overnight. Cells were harvested by centrifugation at 5000 rpm and 4 °C for 15 min, and the pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl. The cells were lysed by sonication, and cell debris was pelleted by centrifugation at 15 000 rpm and 4 °C for 20 min. Approximately 2.5 mL of Ni−NTA−agarose resin (Qiagen) was added per liter of original culture, and binding was performed for 1 h at 4 °C on a rotating platform. After centrifugation at 1500 rpm and 4 °C for 10 min, the resin was resuspended in 20 mL of buffer, washed for 0.5 h, and centrifuged. Washing was repeated two further times. The protein was then eluted from the resin in three steps, each with 4 mL of 250 mM imidazole in 50 mM sodium phosphate buffer (pH 7.5). The tag was cleaved overnight at 4 °C with 30 units of thrombin per liter of original culture. The sample was loaded on a Pharmacia HiLoad 26/60 Superdex 75 column equilibrated with 50 mM phosphate buffer (pH 7.5), 300 mM NaCl, and 1 mM EDTA (to prevent metal-mediated oligomers from forming). For the wild-type proteins, fractions at an elution volume corresponding approximately to the monomer were pooled and dialyzed versus 5 mM Tris buffer (pH 7.5) and 1 mM EDTA. The samples were pure as judged by SDS−PAGE and mass spectrometry. The mutant EP63 proteins were expressed and purified in the same way as the wild type. The protein concentration was determined spectroscopically using the method of Gill and Von Hippel (*21*).

Urea-Induced Equilibrium Denaturation. Aliquots (0.8 mL) of urea solutions were prepared by dispensing the appropriate volumes of a concentrated solution of denaturant in buffer, and buffer alone, using a Hamilton MicroLab M instrument. Protein stock (100 μL) was then added to a final concentration of 4 μM. The samples were equilibrated at the required temperature for 1 h before measurement. Fluorescence measurements were taken on an Aminco Bowman Series 2 luminescence spectrometer. An excitation wavelength of 280 nm was used, and the excitation and emission bandwidths were 4 nm. Wavelength scans between 300 and 370 nm were performed for each sample at a scan speed of 1 nm/s. The cell was thermostated using a water bath, and the temperature of each sample was monitored before measurement using an Edale Instrument thermometer.

CD spectra were recorded on a Jasco J720 spectropolarimeter using a cell with a path length of 0.05 cm. The protein concentration was 20 μM. Spectra were acquired at a scan speed of 1 nm/s with a 1 nm slit and a 0.2 s response time.

It is usually assumed that there is a linear relationship between the free energy of unfolding in the presence of denaturant (abbreviated here to *D*) and the concentration of denaturant (*22*, *23*):

$$\Delta G_{U-F}{}^{D} = \Delta G_{U-F}{}^{H_2O} - m[D] \quad (1)$$

Table 1: Kinetic Parameters for ckshs1 and ckshs2[a]

	ckshs1	ckshs2
$k_u{}^{H_2O}$ (s^{-1})	0.9 ± 0.3	1.4 ± 0.6
$m_u{}^{H_2O}$ (M^{-1})	1.22 ± 0.10	1.08 ± 0.04
m_u* (M^{-2})	−0.06 ± 0.02	−0.05 ± 0.02
$k_{1refolding}$ (s^{-1})	148 ± 2	83 ± 1
$k_{2refolding}$ (s^{-1})	9.4 ± 2.3	6.7 ± 0.8
$k_{3refolding}$ (s^{-1})	0.50 ± 0.11	0.14 ± 0.01
A_1 (%)	88.0 ± 0.5	87.7 ± 0.1
A_2 (%)	5.3 ± 0.5	2.2 ± 0.1
A_3 (%)	6.7 ± 0.6	10.1 ± 0.5

[a] The data are the average of repeat measurements. A_1, A_2, and A_3 are the relative amplitudes of the three phases, observed for refolding.

where $\Delta G_{U-F}{}^{D}$ is the free energy of unfolding at a particular denaturant concentration, [D], $\Delta G_{U-F}{}^{H_2O}$ is the free energy of unfolding in water, and *m* is a constant that is proportional to the increase in the degree of exposure of the protein on denaturation. From eq 1, it is apparent that at $[D]_{50\%}$, the concentration of denaturant at which 50% of the protein is denatured, $\Delta G_{U-F}{}^{H_2O} = m[D]_{50\%}$; thus

$$\Delta G_{U-F}{}^{D} = m([D]_{50\%} - [D]) \quad (2)$$

The denaturation curves were fitted to an equation, derived from eq 2 above (*24*), which yields the values for $[D]_{50\%}$ and *m* and their standard errors (Table 1). Fitting was performed by nonlinear least-squares analysis using the general curve fit option of the program Kaleidagraph (Abelbeck Software).

Refolding and Unfolding Kinetics. Kinetic experiments were performed using an Applied Photophysics fluorescence-detected stopped-flow instrument. The protein concentration was 2 μM unless stated otherwise. Kinetic unfolding experiments were performed by mixing protein in 50 mM Tris buffer (pH 7.5) and 1 mM EDTA with 10 volumes of a urea solution containing the same buffer. pH-jump refolding was performed by mixing protein denatured in 30 mM HCl with an equal volume of renaturing buffer [100 mM Tris (pH 8.1) and 2 mM EDTA] to give a final buffer of 50 mM Tris (pH 7.5) and 1 mM EDTA. Refolding using the urea-jump method was performed by mixing denatured protein in 7.7 M urea, containing 50 mM Tris buffer (pH 7.5) and 1 mM EDTA, with 10 volumes of renaturing buffer [50 mM Tris (pH 7.5) and 1 mM EDTA]. The fluorescence was monitored above 320 nm using a cutoff filter. A minimum of three scans was collected at each denaturant concentration, and the average was fitted using the program Kaleidagraph.

Analytical Size-Exclusion Chromatography. Protein samples were dialyzed into the required buffer and concentrated where required using Vivaspin 20 concentrators with a molecular mass cutoff of 5 kDa (Vivascience). The samples were loaded on a Superdex 75 HR10/30 analytical gel filtration column (Pharmacia) connected to a Pharmacia Åkta system. The column was equilibrated and run in the same buffer as that used for the sample, and it was thermostated using a jacket attached to a water bath. The proportions of the different species were determined by integration of the areas of the elution peaks using the Pharmacia UNICORN Evaluation package.

Differential Scanning Calorimetry. Measurements were performed using a MicroCal VP-DSC calorimeter with a cell volume of 0.52 mL. The instrumental scan rate was 1 °C/

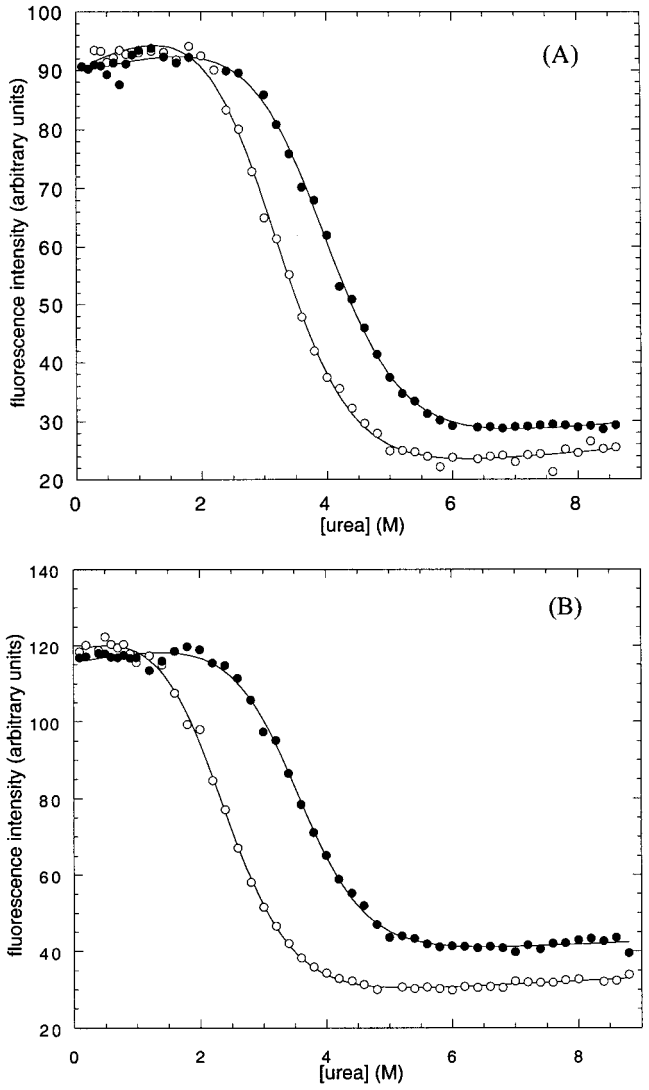

FIGURE 2: Urea-induced denaturation of (A) ckshs1 and (B) ckshs2 in 50 mM Tris buffer (○) and in 50 mM sodium phosphate buffer (●), both at pH 7.5, with 1 mM EDTA at 25 °C. The protein concentration was 4 μM, and the fluorescence intensity is plotted at an emission wavelength of 325 nm.

min. The preparation of samples and the analysis of the data were as described previously (*25*). The loading and operation of the instrument followed the manufacturer's instructions.

RESULTS

ckshs1 and ckshs2 Have Marginal Thermodynamic Stability. The proteins have two tryptophan residues that are partly buried in the structure. The fluorescence spectrum of the native protein upon excitation at 280 nm has a maximum intensity at 335 nm, consistent with the locations of the tryptophans in the structure. There is a large decrease in fluorescence intensity upon unfolding, and a shift in the maximum to 350 nm. ckshs1 and ckshs2 unfold reversibly in urea as shown by the complete recovery of the fluorescence signal upon refolding. Equilibrium denaturation was performed using an excitation wavelength of 280 nm and an emission wavelength of 325 nm, at which the change in fluorescence signal upon unfolding was a maximum (Figure 2). A single structural transition was observed, with midpoints at 3.09 and 2.29 M, respectively. The free energies of unfolding extrapolated to water, $\Delta\Delta G_{U-F}$, were 3.00 and 2.47 kcal/mol, respectively (at 25 °C). The same midpoint of denaturation and *m* value were obtained when the emission was plotted at wavelengths other than 335 nm. The unfolding equilibrium was also monitored by CD. Neither protein exhibits the double minimum in ellipticity at ~222 nm that is typical of helical structure, and this is probably due to the low helical content and/or a positive contribution at this wavelength from tryrosine residues (of which ckshs1 and ckshs2 have five and six, respectively). There is an ellipticity maximum at 235 nm that is characteristic of asymmetric aromatic residues that exhibited the same transition with denaturant as that observed for fluorescence.

Urea denaturation was also performed in phosphate buffer (50 mM, pH 7.5), instead of Tris buffer, because it is known that the cks proteins can bind phosphate ions. The stability of both proteins was enhanced by the addition of phosphate ions, and the same behavior was observed previously for suc1 (*18*), consistent with evidence pointing to the binding of cks proteins to phosphorylated substrates (*26*, *27*). The *m* values were unchanged, but the midpoints of unfolding were shifted by 0.8 and 1.2 M for ckshs1 and ckshs2, respectively, in the presence of 50 mM sodium phosphate. The free energies of unfolding under these conditions are 3.82 and 3.84 kcal/mol, respectively. Differential scanning calorimetry was attempted at protein concentrations in the range between 40 and 80 μM in 50 mM phosphate buffer at pH 7.5; however, the signal was too small at the lower concentration, and the samples aggregated at the higher concentration. Aggregation was also observed when thermal denaturation was monitored by fluorescence at low protein concentrations of 4 μM.

Kinetics of Unfolding. Unfolding was initiated by diluting the folded protein into different concentrations of urea in a stopped-flow apparatus, and it was monitored by fluorescence. For both proteins, there was a decrease in fluorescence, and the kinetics could be fitted to a single-exponential process (Figure 3). The urea dependence of the logarithm of the rate constant shows slight downward curvature at high concentrations of denaturant, and the data outside the transition region fit well to a second-order polynomial of the type

$$\ln k_u = \ln k_u^{H_2O} + m_u[D] + m_u^*[D]^2 \qquad (3)$$

where k_u is the rate constant for unfolding at a denaturant concentration, [D], and $k_u^{H_2O}$ is the rate constant of unfolding in water. m_u is the *m* value that reflects the change in solvent exposure between the native state and the rate-determining transition state for unfolding. Similar nonlinearity has been observed for a number of other proteins, and has been interpreted as a result of movement of the transition state for unfolding according to Hammond behavior, and rigorously tested with mutations in the protein barnase (*28*). The values of $k_u^{H_2O}$, m_u, and m_u^*, determined using eq 3, are given in Table 1. The unfolding kinetics were independent of protein concentration in the range of 1−100 μM.

Upon Refolding, ckshs1 and ckshs2 Transiently Populate Oligomeric Species That Slow the Reaction. Refolding was monitored by dilution of acid-denatured protein into buffer containing different concentrations of urea. In the absence of denaturant, three distinct phases were observed, a major fast phase and two minor slower phases. The rate constants

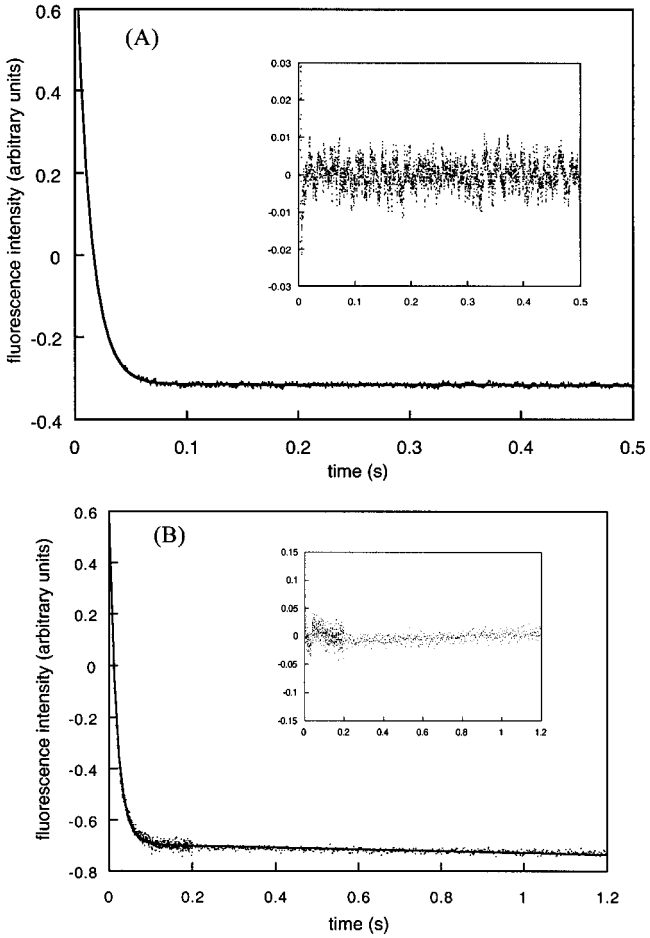

FIGURE 3: Typical kinetic trace for unfolding of (A) ckshs1 and (B) ckshs2. Insets show the residuals for a fit to a single-exponential function.

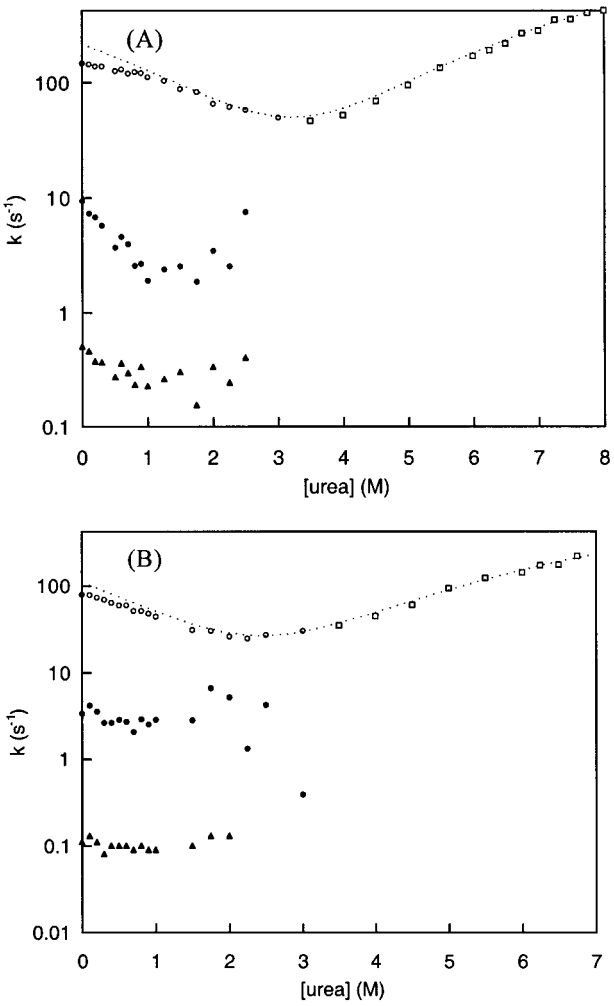

FIGURE 4: Urea dependence of the refolding and unfolding kinetics of (A) ckshs1 and (B) ckshs2. Experiments were performed in 50 mM Tris buffer at a final pH after mixing of 7.5, with 1 mM EDTA at 25 °C. The protein concentration after mixing was 2 μM. Empty circles represent the major refolding phase and filled circles and triangles the slower and slowest minor refolding phase, respectively. Unfolding data are shown with squares. The dotted lines show the rate constant expected for two-state behavior.

and their relative amplitudes are given in Table 1. The same kinetics were observed when 2 M urea was present in the acid-denatured protein, and also for refolding of the urea-denatured protein.

The denaturant dependence of the natural logarithm of the observed relaxation rates is shown in Figure 4. The kinetics were triphasic at all the denaturant concentrations that were used. The rate constant for the fastest phase decreases steeply and approximately linearly with increasing denaturant concentration. However, the observed rate constant at low urea concentrations deviates slightly from that expected from two-state behavior, as calculated by combining the equilibrium and kinetic unfolding data (Figure 4).

The refolding reaction became markedly slower when the protein concentration was increased (Figure 5A). When the data were fitted to the sum of three exponential phases, the rate constant of the fastest phase decreased considerably while that of the slower phase decreased and that of the slowest phase remained approximately unchanged (Figures 5B and 6A). As the protein concentration was increased, the amplitude of the fastest phase decreased and that of the slower phase increased (Figures 5C and 6B). The data were also refitted with the rate of the slower phase fixed to a constant value (25 s^{-1} in the case of ckshs1 and 15 s^{-1} in the case of ckshs2) throughout the concentration range. The rate constant of the fast phase then remained approximately constant over the whole concentration range; the amplitude pattern was the same as that obtained when the data were fitted without any fixing of parameters. Thus, the overall reaction is retarded with increasing protein concentrations as a result of the increasing amplitude of the slower phase. The results suggest that there is partitioning between fast folding directly to the native state and a slower process involving formation of a transient oligomeric species that must unfold before refolding to the native state. The plots of the relative amplitudes of the kinetic phases versus protein concentration show that, even at concentrations of <1 μM, some oligomerization occurs. This probably accounts for the small deviation of the refolding rate constants from those expected for two-state behavior. The protein concentration dependence of refolding was monitored in the presence of increasing concentrations of urea, below the unfolding transition region. Even when urea, which is expected to dissociate oligomeric species, was present in the refolding buffer, there was a marked retardation of the fast refolding rate constant with increasing protein concentrations. When refolding was performed from urea-denatured protein, a similar protein concentration-dependent retardation of the kinetics was also observed (data not shown).

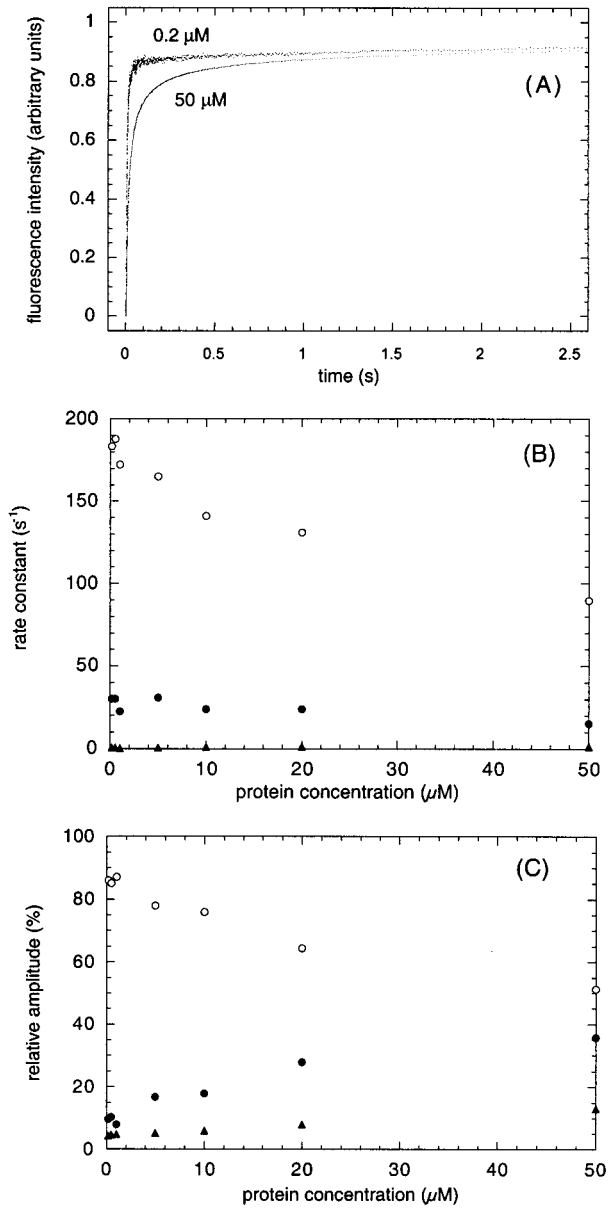

FIGURE 5: Dependence on protein concentration of the refolding kinetics of ckshs1. (A) Kinetic trace for refolding at protein concentrations of 0.2 and 50 μM. Only the first few seconds of the reactions are shown to highlight the retardation at the higher protein concentration. (B) Amplitudes and (C) rate constants of the three refolding phases as a function of protein concentration. The buffer conditions were the same as those described in the legend of Figure 4. The temperature was 10 °C.

To determine whether oligomers are present in the denatured state prior to the initiation of refolding, NMR and size-exclusion chromatography experiments were performed. Protein samples at a concentration of 100 μM in 6 M urea showed no evidence of line broadening in the NMR spectra that would be indicative of high-order species. Further, a single peak was observed by size-exclusion chromatography (Pharmacia analytical S200 column) at an elution volume of ~15.3 mL for a sample in 6 M urea. This volume corresponds to that observed for other monomeric denatured proteins of a similar size. Finally, there is no evidence of a protein concentration dependence of the equilibrium unfolding of ckshs1 or ckshs2. If the denatured state aggregated, this would shift the equilibrium between folded and denatured protein and consequently the midpoint of unfolding would appear to be lower upon increasing the protein concentration.

ckshs2 Associates into Domain-Swapped Dimeric and Hexameric Species, while ckshs1 Forms Only Dimers. The crystal structure of ckshs2 revealed a domain-swapped dimer that associated further into a hexamer. ckshs1 has only been observed previously as a monomer. We have estimated the dissociation constants of the oligomeric forms of the human cks proteins using analytical size-exclusion chromatography. The samples were equilibrated at 10 °C since higher temperatures resulted in aggregation. Measurements were taken at 4 °C with a column thermostated using a cooling water bath. The crystal structure of the ckshs2 hexamer suggested that the dimer would not be very stable due to the proximity of residue E63 in the two monomers. This effect is balanced by anion binding that stabilizes the (non-domain-swapped) association in the hexamer. Therefore, we used phosphate buffer at pH 6.8 (50 mM) to enhance oligomerization. In the absence of phosphate at this pH, neither dimer nor hexamer forms are observed. We have also looked at a mutant, EP63, in both proteins. The residue is located in the hinge loop which mediates domain swapping in the cks proteins, and mutation to proline at the equivalent position in suc1 greatly increases its domain swapping propensity (*20*).

The elution profile obtained for wild-type ckshs2 showed a major peak corresponding to the monomer and two minor peaks at volumes expected approximately for the dimer and hexamer (Figure 7). For wild-type ckshs1, only a very small peak (<5%) was observed at a volume corresponding to the dimer, the major peak being at the monomer volume, and no hexamer could be detected. This was the case even at protein concentrations of 1−10 mM. Elution profiles of ckshs2 EP63 revealed proportions of dimer and hexamer much higher than that observed for the wild type (peaks of approximately equal volumes were observed at a protein concentration of ~200 μM). Similarly, for ckshs1 EP63 at a concentration of ~9 mM, monomer and dimer peaks of approximately equal volumes were observed. In each case, the elution volume of the monomer in the mutant run was slightly smaller than that observed in the corresponding wild-type run. This suggested that dissociation of the dimer occurs on a time scale similar to that of the chromatography. Consistent with this, when the dimer peak of any of the proteins was collected and re-injected onto the column immediately after the end of a run, two peaks were observed at approximately the monomer and dimer volumes. Consequently, it was not possible to determine accurately the values of K_d using size-exclusion chromatography. This requires a technique such as analytical ultr-centrifugation, and these experiments are currently underway.

DISCUSSION

Nature of the Equilibrium Oligomeric Species. The association behavior of ckshs1 and ckshs2 was probed at equilibrium using the wild-type proteins and the mutants EP63. Residue 63 is in the loop that acts as a molecular hinge effecting domain swapping in the cks proteins. Mutation of the proline at the equivalent position in suc1 greatly increases its domain swapping propensity (*20*). The explanation is as follows. There are two conserved proline residues in the

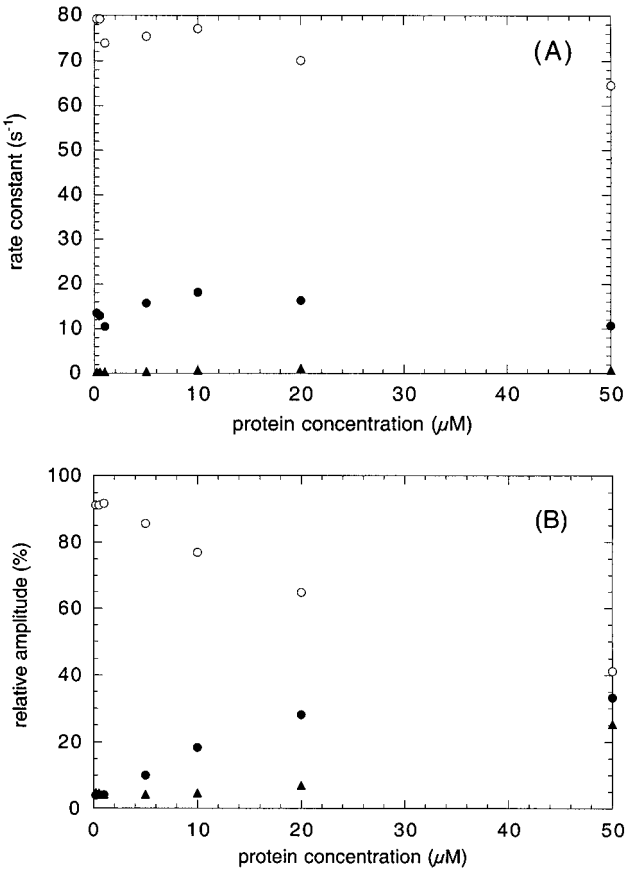

FIGURE 6: Dependence on protein concentration of the refolding kinetics of ckshs2. (A) Amplitudes and (B) rate constants of the three refolding phases as a function of protein concentration. The buffer conditions were the same as those described in the legend of Figure 4. The temperature was 10 °C.

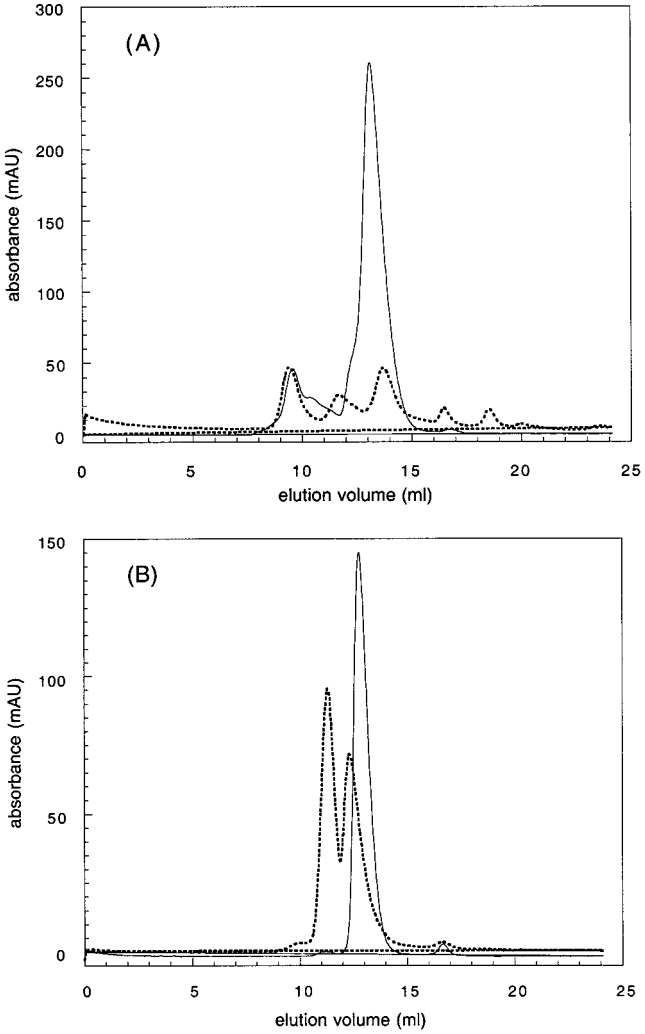

FIGURE 7: (A) Size-exclusion chromatogram of wild-type ckshs2 at a concentration of 1.8 mM (—) and EP63 at 180 μM (- - -). (B) Size-exclusion chromatogram of wild-type ckshs1 at a concentration of 1.0 mM (—) and EP63 at 8.8 mM (- - -). The running buffer was 50 mM phosphate (pH 6.8) and 300 mM NaCl, and the temperature was 4 °C.

hinge loop sequence of the cks proteins. Introducing a third strains the turn conformation that is adopted in the monomer form and thus favors the extended conformation of the hinge loop in the dimer form. The equilibrium between the monomer and dimer can also be shifted toward the dimer by introducing strain into the monomer hinge loop conformation using other means such as shortening it, thereby making it more difficult for it to fold back on itself. Such an effect has been shown for a number of proteins (*16*, *20*, *29*, *30*).

Without a structure, we cannot be certain that the ckshs1 dimer is domain-swapped, but this is the simplest conclusion that can be drawn from our results. The greater proportion of dimeric species observed in the Glu → Pro mutant compared with the wild type in all three cks proteins suggests strongly that the dimer is domain-swapped. We know from our studies on suc1 that this mutation has a highly specific effect on domain-swapped association, whereas there is nothing to indicate that the mutation could increase the level of oligomerization by some other, non-domain swapping process.

The domain swapping propensity of ckshs2 appears to be significantly greater than that of ckshs1. Interestingly, only dimers were observed for ckshs1, while both dimers and hexamers were observed for ckshs2. There is no obvious explanation from the crystal structures for the different behavior of the two proteins, and it is surprising in view of their highly identical sequences. The relative proportion of dimer and hexamer species in ckshs2 EP63 was different from that observed for the wild type, with more dimer than hexamer being formed in the mutant protein. This is consistent with the observation that the wild-type dimer is destabilized by the proximity of the glutamates at position 63.

Nature of the Transient Oligomers. Oligomeric species are formed transiently upon refolding of ckshs1 and ckshs2 proteins at concentrations in the micromolar range. Importantly, the end product of the reactions is monomeric in this range (as determined by loading the solutions on a gel filtration column within minutes of initiating the folding reaction). The reversibility and reproducibility of the aggregation processes upon refolding of the cks proteins suggest that these are specifically ordered states. Similar behavior was observed previously for the refolding of two other small proteins, CI2 and U1A (*31*). As pointed out by these authors, native contacts are likely to be more favorable in folding than non-native ones, and we already know that the exchanging β-strand 4 can make interactions intermolecularly (in the domain-swapped dimer or hexamer) as well as intramolecularly. Therefore, association to form higher-order species

could also occur via intramolecular overlap of the complementary surfaces that are found in the domain-swapped forms of these proteins. It is difficult to probe directly the transient species using structural tools, but mutant studies aimed at determining whether there is a correlation between the domain swapping propensity and the extent of oligomerization may help to address this point.

Comparison of the Behavior of Human and Yeast cks Proteins. The two human cks proteins have a strong tendency to form transient oligomers upon refolding from the denatured state. Whether these species are domain-swapped remains to be determined. At equilibrium and under native conditions, the tendency to form domain-swapped states is very weak, although the aggregation propensity even at room temperature is high. The proteins also exhibit low thermodynamic and kinetic stabilities and lower melting temperatures, and these properties probably explain the observed aggregation tendency. The K_d for the domain-swapped dimer of suc1, the cks from *S. pombe*, is also high, in the millimolar range (*20*). However, unlike the human proteins, suc1 is relatively stable to both chemical and thermal denaturation, and it unfolds several orders of magnitude more slowly than ckshs1 and ckshs2 (*18*). Further, it aggregates only at elevated temperatures. These characteristics might be expected from its larger size; a much more extensive hydrophobic core results from packing of the additional secondary structure elements onto the β-sheet.

suc1 refolds via an intermediate, which, again, is likely to reflect the larger size of the protein compared with the human homologues. However, the intermediate in suc1 does not have the type of structure that might be expected for large proteins with more than one subdomain (*19*). It does not consist of some subdomains that are fully folded and other subdomains that are fully unfolded, nor does it have folded subdomains that have not yet docked onto each other. Instead, both secondary and tertiary interactions are partly formed in the intermediate. Therefore, the reason for the absence of intermediates in the human proteins cannot be the lack of distinct subdomains that have some autonomous stability, although it is likely that the additional structural elements that are present in suc1 do stabilize partly folded intermediate states.

We have previously proposed that interconversion between the monomer and the domain-swapped dimer in suc1 occurs via the denatured state (*20*). Accordingly, interconversion is faster under conditions that accelerate unfolding. That this model holds generally for the cks family is supported by our findings here. The dissociation of the domain-swapped state is faster for ckshs2 than for suc1, consistent with the much faster unfolding rate of the former protein.

The different biophysical properties of the human cks proteins when compared with those of suc1 contrast with their apparently identical functions in cdk regulation in the cell. Indeed, both human proteins can substitute for cks in yeast (*12*). Interestingly, however, neither ckshs2 nor suc1 was able to replace ckshs1 in its recently discovered role in the G1−S transition (*3*, *4*). The level of sequence identity within the cks family is very high, and the only difference between the human proteins and the *S. pombe* protein is their sizes. suc1 (and the cks from *Sa. cerevisiae*) has two large insertions, a long α-helix at the N-terminus and a long loop, both of which contribute additional packing residues to the hydrophobic core. The different behavior of these proteins illustrates the balance of interactions that arises in protein folding. Transient oligomerization during refolding of ckshs1 and ckshs2 occurs at somewhat lower protein concentrations than that of suc1 (unpublished results). This may be related to the presence of an intermediate in the folding reaction of suc1. If the oligomers form from the denatured state and there is kinetic competition between oligomerization and refolding, then the slower the folding reaction the greater the likelihood of oligomerization occurring (assuming that the rate of aggregation is the same for the yeast and human proteins). Folding of suc1 is via the rapid formation of an intermediate [$k \sim 1000\ s^{-1}$ (*18*)], whereas folding of ckshs1 and ckshs2 occurs directly from the denatured state to the native state at a rate that is approximately 1 order of magnitude slower.

Domain Swapping Mechanism in the cks Proteins. The propensities of the human and yeast cks proteins to form domain-swapped oligomers are similar, suggesting that this process is controlled by a common feature of the proteins. A likely candidate is the conserved Pro-X-Pro motif in the hinge loop that we have shown previously to have a dominant effect on the position of the monomer−dimer equilibrium in suc1 (*20*). Proline repeats have been identified in the hinge region of a number of domain-swapped proteins (*32*). The presence or absence of partly folded intermediates appears to have no effect on the domain swapping behavior of these proteins, indicating that such a state is not required as a precursor to the association. This is again consistent with the requirement for complete unfolding for domain swapping to occur. Thus, the ability of any protein to undergo domain swapping is unlikely to be determined by a global feature of the protein, such as size, the presence of separate domains, or multistate folding.

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