Folding and conformational studies on SCR1–3 domains of human complement receptor 1

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Introduction

All proteins must adopt specific three-dimensional structures, known as the native or fully folded state, in order to be biologically active. For most proteins all the information required to define the native state is contained within the amino acid sequence and folding occurs spontaneously under appropriate conditions. If a protein were to sample all possible conformations randomly until the correct structure was encountered, folding would take considerably longer than is actually required. This fact suggests that protein folding must be directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993). It is generally believed that protein folding occurs through intermediate, partially-directed in some way (Creighton, 1993)
spectroscopy were used to follow conformational changes in the proteins. Stopped-flow fluorescence experiments were also performed to determine the kinetics of folding and to attempt to detect any intermediate species.

Materials and methods

Expression and purification of SCR1–3
SCR1–3 was expressed and purified using the method described by Dodd et al. (1995) and SCR3 was prepared as described by Clark (1996).

Assay
The functional activities of SCR1–3 were assessed by the ability to inhibit complement-mediated lysis of red blood cells (Dodd et al., 1995). The inhibition by SCR1–3 is ~400-fold less than that given by intact CR1 (mol:mol), which has 30 consecutive SCRs.

Spectroscopy
A Hitachi F-2000 fluorescence spectrophotometer and a Jasco J-720 CD spectropolarimeter, each with jacketed cell holders connected to circulating water-baths, were used in the equilibrium studies. Kinetic data were obtained using an Applied Photophysics stopped-flow spectrofluorimeter.

Folding/unfolding of SCR3 and SCR1–3 using circular dichroism and fluorimetry
GdnHCl solutions were prepared over a concentration range 0–7.5 M in 20 mM phosphate buffer, pH 7.4. For fluorescence analysis, 50 µl of a 0.8 mg/ml SCR3 or SCR1–3 stock solution in 20 mM phosphate, pH 7.4, was diluted into 1 ml of the appropriate denaturant concentration to give a final protein concentration of ~40 µg/ml. The fluorescence was excited by an incident beam at 280 nm and the emission intensity was monitored at 338 nm.

For far-UV CD analysis, 50 µl of stock SCR1–3 solution (4.0 mg/ml) was diluted into 450 µl phosphate buffer to give a final protein concentration of 0.4 mg/ml; for near-UV CD analysis the final protein concentration was 2 mg/ml. All samples were pre-equilibrated at the specified temperatures for ~1 h before spectroscopic measurements were carried out in thermostatted cuvette holders, also pre-equilibrated at the specified temperatures. The pH of the phosphate buffer was checked at each of the temperatures used in the experiments.

Unfolding of reduced SCR1–3 was performed as described above, with the addition of 50 mM β-mercaptoethanol to all solutions (pH 7.4). All fluorescence data were corrected for the inner filter effect by the equation $F_{obs} = F_{corr} \times 10^{0.004}$ (Miller, 1981), where $A$ represents the absorbance of the solution at the excitation wavelength, in a cell of 1 cm light path.

Kinetics
Unfolding of SCR3 or SCR1–3 (~0.1 mg/ml in 20 mM phosphate, pH 7.4) was initiated by 1:10 mixing with various GdnHCl solutions to give a final range between 2 and 7 M. The reaction progress curves describing unfolding/refolding were analysed by single- and double-exponential algorithms in software provided with the instrumentation. Refolding was performed by 1:10 v/v mixing of SCR3 or SCR1–3 in 3–7 M GdnHCl with solutions containing various GdnHCl concentrations to give a final range between 0.27 and 4 M. All kinetic data were obtained at 25°C unless stated otherwise.

Data analysis

GdnHCl denaturation
The data analysis assumes a two-state model of unfolding, i.e. an equilibrium between folded and unfolded protein. The transition region between the two extremes yields equilibrium constants, $K_{eq}$, for unfolding or folding from which the free energy of unfolding, $G_U$, can be calculated:

$$G = -RT \ln K_{eq}$$  \hspace{1cm} (1)

The free energy of unfolding for a protein is linearly related to the denaturant concentration (Pace, 1986):

$$G_U = G_{H_2O} - m[\text{denaturant}]$$  \hspace{1cm} (2)

where $G_{H_2O}$ is the difference in free energy between the folded and unfolded conformations in water, representing the conformational stability of the protein, and $m$ is a measure of the dependence of $G$ on GdnHCl concentration.

Unfolding in the presence of GdnHCl at various temperatures
A series of GdnHCl unfolding experiments was performed as described previously using fluorimetry, at a range of temperatures between 20 and 49°C in order to calculate the $K_{eq}$ of unfolding as a function of temperature. Fluorescence data were plotted as a percentage of the maximum fluorescence obtained, which represented fully unfolded protein. The change in enthalpy associated with unfolding ($\Delta H$) can be calculated from the van’t Hoff equation:

$$d(\log K_{eq})/d(1/T) = -\Delta H/2.303R$$  \hspace{1cm} (3)
GdnHCl 7 M was used to denature the protein fully and 50 mM β-mercaptoethanol to reduce the six disulphide bonds present in SCR1–3. Reduced native; (—) non-reduced native; (•••) reduced denatured; non-reduced, native and denatured SCR 1–3 at pH 7.4 (40 Hg/ml). Spectra obtained at an excitation wavelength of 280 nm for reduced and unfolded protein there is a difference in heat capacity, $AC_p$:

$$\Delta C_p = C_p(U) - C_p(F)$$

where $C_p(U)$ and $C_p(F)$ are the heat capacities of the unfolded and folded conformations, respectively, and $\Delta C_p$ is the change in heat capacity that accompanies protein unfolding.

$\Delta H$ is not constant but varies with temperature because the heat capacity of the unfolded protein is greater than that of the folded protein. For the reaction between folded and unfolded protein there is a difference in heat capacity, $AC_p$:

$$d(\Delta H)/dT = \Delta C_p$$

**Enthalpy and entropy of activation**

The enthalpy and entropy of activation can be determined using the Eyring (1935) equation:

$$k = (k_B T \kappa h) \exp(\Delta S^*/R) \exp(-\Delta H^*/RT)$$

where $k$ is the rate constant, $T$ is the absolute temperature, $\Delta H^*$ and $\Delta S^*$ are the enthalpy and entropy of activation, respectively, $k_B$ is the Boltzmann constant, $\kappa$ is the Planck constant and $h$ is the transmission coefficient which, being close to 1, is ignored. Hence, according to the transition state theory, a plot of $\ln(k/T)$ vs $1/T$ has a slope of $-\Delta H^*/R$ and an intercept on the y-axis equal to $\ln(k_B h) + \Delta S^*/R$.

**Results**

**Studies on the three-domain protein SCR1–3**

**Fluorescence and CD spectroscopy.** Figure 2 illustrates the fluorescence spectra for reduced and non-reduced, folded and unfolded SCR1–3 at pH 7.4. The fluorescence spectrum of native, non-reduced SCR1–3 displays an emission maximum at 338 nm with no detectable contribution from the eight Tyr residues in SCR1–3, suggesting that resonance energy transfer occurs between these and the four Trp residues (Lakowicz, 1983). Upon reduction of the six disulphide bridges by the addition of 50 mM β-mercaptoethanol, the intensity of the fluorescence emission increases approximately twofold although the wavelength of maximum emission remains at 338 nm. Unfolding of the non-reduced protein by the addition of 7 M GdnHCl leads to an ~13.7-fold increase in fluorescence signal with a new wavelength of maximal emission at 348 nm. The additional presence of 50 mM β-mercaptoethanol does not lead to a further increase in the fluorescence intensity.

Figure 3 illustrates part of the far-UV CD spectra (210–250 nm) for native and denatured SCR1–3. Native SCR1–3 illustrates positive ellipticity between 217 and 242 nm with a negative ellipticity below 215 nm. The spectrum shows little evidence of classical secondary structural elements and appears to be devoid of any α-helicity. This is consistent with previous CD studies on SCR1–3 (Dodd et al., 1995) and NMR analysis of other SCR modules (Barlow et al., 1991, 1992; Norman et al., 1991). Denatured SCR1–3 has weak negative ellipticity from 250 to 225 nm and below this wavelength the ellipticity becomes more negative. The maximum signal change upon denaturation occurred at 234 nm and this wavelength was therefore used to follow unfolding of the protein. Spectral analysis of SCR1–3 in the near-UV region (250–300 nm) indicated that the folded protein has a negative ellipticity with a minimum at 283 nm, most probably attributable to the aromatic amino acid side chains in a chiral environment (see inset to Figure 3). The presence of GdnHCl leads to the ellipticity approaching zero at these wavelengths reflecting more mobility and disruption of the packing of the side chains as the protein unfolds.

The unfolding of SCR1–3. In order to examine the conformational stability of the protein, experiments were carried out in which the fluorescence intensity of the protein was measured in various concentrations of denaturant (GdnHCl). The increase in fluorescence intensity of SCR1–3 upon unfolding is unusual since for most proteins unfolding is accompanied by a decrease in fluorescence intensity between 330 and 340 nm. The decrease typically indicates tryptophan residues becoming more exposed to the solvent. Unfolding of SCR1–3 is, however, accompanied by a red shift in its wavelength of maximum emission from 338 to 348 nm. Figure 4 gives typical unfolding curves for SCR1–3 in GdnHCl. The curve shows three distinct phases: below 2 M GdnHCl, the fluorescence intensity remains largely unchanged, indicating that little unfolding occurs; between 3 and 5 M GdnHCl, there is a single transition region between the two extreme states, folded and unfolded; and above 5 M GdnHCl, the fluorescence intensity stabilizes as the fully unfolded form of the protein is approached. The data from the transition region were transformed using equation (1) to give a plot of $G$ against [GdnHCl] shown in the inset to Figure 4. For SCR1–3, the conformational stability or $G_{H_2O}$ at 25°C is 19.5 kJ/mol and $m$ is 4.6 kJ/mol.M. The mid-point of unfolding is found at 4.2 M GdnHCl. In experiments using urea as a denaturant incomplete unfolding occurred even in 9 M urea.

To examine whether the unfolding processes and conformational stability measured by fluorescence spectroscopy are a global and not simply a local representation of unfolding of SCR1–3 in the proximity of the tryptophan residues, GdnHCl titrations were also performed monitoring CD in the near- and far-UV region of the spectrum (see Figure 3). A summary of the results obtained are given in Table I. The average values of $G_{H_2O}$, $m$ and the mid-point obtained from the three
Fig. 3. Far-UV CD spectra of SCR1–3. Folded SCR1–3 in 20 mM phosphate, pH 7.4 (0.4 mg/ml) (curve 1) and unfolded SCR1–3 in 20 mM phosphate, pH 7.4 (0.4 mg/ml) containing 7 M GdnHCl (curve 7). Curves 2–6 were obtained with the same concentration of SCR1–3 in the presence of 3.0, 3.9, 4.2, 4.5 and 5.1 M GdnHCl, respectively. Inset: near-UV CD spectra of SCR1–3. The spectra of 2 mg/ml folded (1) or unfolded (4) SCR1–3 in the same buffer system as above. Curves 2 and 3 were obtained in the presence of 3.3 and 4.2 M GdnHCl, respectively.

![Graph showing fluorescence at 338 nm against [GdnHCl] molar](image)

Fig. 4. GdnHCl-induced unfolding of non-reduced SCR1–3 and SCR3 at 25°C. SCR1–3 (●) or SCR3 (■) (40 µg/ml) was dissolved in 20 mM potassium phosphate buffer, pH 7.4. The fluorescence intensities at 338 nm are shown as a function of GdnHCl concentration. Inset: fluorescence data from the transition region of Figure 3 were used to calculate K_m and hence changes in ΔG from equation (1). The calculated values of ΔG are plotted against the concentration of the denaturant. Extrapolation of the curve to 0 M GdnHCl yields the change in free energy of unfolding in the absence of denaturant, ΔG_H2O.

Table I. Summary of the results (ΔG_H2O, m and midpoint) obtained for unfolding studies of reduced and non-reduced SCR1–3 using tryptophan fluorescence, near- and far-UV circular dichroism

<table>
<thead>
<tr>
<th>Technique</th>
<th>Reduced/oxidized</th>
<th>ΔG_H2O (kJ/mol)</th>
<th>m (kJ/mol.M)</th>
<th>Mid-point [GdnHCl] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
<td>S–S–</td>
<td>19.5</td>
<td>4.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Near-UV CD</td>
<td>S–S–</td>
<td>19.5</td>
<td>5.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>S–S</td>
<td>19.9</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Average</td>
<td>S–S–</td>
<td>19.6 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>SH HS–</td>
<td>9.8</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Far-UV CD</td>
<td>SH HS–</td>
<td>11.2</td>
<td>4.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The unfolding of SCR1–3 was also examined in the presence of 50 mM β-mercaptoethanol in order to follow the unfolding transition in the absence of disulphide bridges. Both spectroscopic techniques showed that under reducing conditions the protein is less stable, the mid-point of the unfolding curves moving to lower concentrations of denaturant. The decrease in the values of G_H2O determined were due to lower values of the mid-points of the transitions (see Table I).

The unfolding of SCR1–3 in GdnHCl was carried out over a range of different temperatures between 20 and 49°C. At 49°C the mid-point of the transition was reached when 2.5 M
GdnHCl was present, whereas at 20°C 4.4 M denaturant was required. The higher the temperature, the lower was the concentration of denaturant required to unfold the protein (see Figure 5). Inset (a) to Figure 5 shows the corresponding van't Hoff plots using data derived from Figure 5 from which \( \Delta H \) can be calculated according to the relationship described by equation (3). The curves shown were obtained at GdnHCl concentrations of 3.0 and 4.0 M. These were examples of concentrations chosen for analysis because they enabled equilibrium constants to be obtained within the transition region of unfolding over a wide range of temperatures. Inset (b) to Figure 5 shows the corresponding relationship between \( \Delta H_f \) and temperature from which \( \Delta C_p \) of unfolding can be calculated from equation (5). The estimate obtained for \( \Delta C_p \) of unfolding was 6.6 kJ/K.mol or 33.8 J/K.mol.

**Kinetics of SCR1-3 folding and unfolding**

Figure 6 illustrates data obtained by stopped-flow fluorescence experiments to measure the rates of unfolding or refolding of non-reduced SCR1-3 at 25°C. The refolding process was determined as described under Materials and methods. The individual reaction progress curves in each direction could be fitted to double-exponential equations. Unfolding in 7 M GdnHCl has a fast phase corresponding to >93% of the signal change with a rate constant of 38.8 s\(^{-1}\) and a slow phase corresponding to only <7% of the signal change with a rate constant of 5.6 s\(^{-1}\). The rate of the fast phase of unfolding was found to be dependent upon denaturant concentration, whereas that of the slow rate appeared to be independent. Refolding into 0.63 M GdnHCl has a fast phase (~80% of the signal change with a rate constant of 52.8 s\(^{-1}\)) and a slow phase (up to 20% of the signal change with a rate constant of 6.7 s\(^{-1}\)). Both fast and slow rates of refolding are dependent upon denaturant concentration (see Table II).

The fast phases of unfolding and refolding which are represented by the major signal changes occurring during these processes were used in the subsequent analyses detailed below. The natural logarithms of the rate constants for unfolding and refolding into different GdnHCl concentrations were plotted against GdnHCl concentration (Figure 6). Unfolding shows a linear relationship with concentration of denaturant used; however, refolding clearly deviates from a linear relationship at concentrations of denaturant below 2 M where a rate-limiting step in the process becomes clearly evident. The midpoint of the transition region between rates of unfolding and refolding is ~3.9 M GdnHCl, which is consistent with the midpoint of unfolding obtained during equilibrium experiments described above.

Refolding of fully reduced/denatured SCR1-3 into 20 mM ethanolamine, 1 mM EDTA at 6°C, followed using fluorescence spectroscopy, was shown to proceed at a considerably slower rate than non-reduced SCR1-3 (data not shown). The major structural changes of folding occurred over ~40 min (cf. ~0.05 s for non-reduced SCR1-3 at 25°C with a first-order rate of ~5.8×10\(^{4}\) s\(^{-1}\)). This process was only noted in the presence of a glutathione redox mixture to aid disulphide

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**Table II. The rates of unfolding and refolding of SCR1-3 at various concentrations of GdnHCl**

<table>
<thead>
<tr>
<th>[GdnHCl] (M)</th>
<th>Rate 1 (s(^{-1}))</th>
<th>Rate 2 (s(^{-1}))</th>
<th>[GdnHCl] (M)</th>
<th>Rate 1 (s(^{-1}))</th>
<th>Rate 2 (s(^{-1}))</th>
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<tbody>
<tr>
<td>4.3</td>
<td>13.1</td>
<td>3.6</td>
<td>0.6</td>
<td>52.8</td>
<td>6.5</td>
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<td>4.6</td>
<td>16.7</td>
<td>4.3</td>
<td>1.2</td>
<td>56.7</td>
<td>6.4</td>
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<tr>
<td>4.9</td>
<td>18.0</td>
<td>5.2</td>
<td>1.5</td>
<td>59.3</td>
<td>5.7</td>
</tr>
<tr>
<td>5.4</td>
<td>18.7</td>
<td>5.9</td>
<td>1.8</td>
<td>53.6</td>
<td>4.6</td>
</tr>
<tr>
<td>5.8</td>
<td>20.4</td>
<td>4.6</td>
<td>2.1</td>
<td>42.1</td>
<td>3.4</td>
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<tr>
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<td>26.3</td>
<td>4.4</td>
<td>2.5</td>
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<tr>
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<td>3.7</td>
<td>3.3</td>
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</tr>
<tr>
<td>7.0</td>
<td>38.8</td>
<td>5.6</td>
<td>3.7</td>
<td>9.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Studies on the single-domain protein SCR3

Unfolding at equilibrium. Experiments to compare the unfolding at equilibrium of SCR3 with those of SCR1-3 were carried out to clarify whether the folding characteristics of an individual domain are perturbed by the presence of neighbouring domains. CD studies showed that the SCR3 protein has far-UV spectral characteristics identical with those of SCR1-3 except that the molar ellipticities at all wavelengths are 2.7-fold less than those of SCR1-3 (which equals the ratio of their molecular weights); there is no evidence of the presence of classical secondary structures such as α-helices. Furthermore, the presence of denaturant results in similar spectral changes to those noted for SCR1-3, facilitating unfolding studies at equilibrium. These showed that the conformational stability of the single domain was ~12.1 kJ/mol.

A similar value (13 kJ/mol) was obtained by denaturation studies monitoring the increase in protein fluorescence intensity at 338 nm to follow the unfolding process. The unfolding curve for SCR3 in Figure 4 shows that the unfolding transition takes place at much lower concentrations of denaturant (1–3 M GdnHCl) than those for SCR1-3. Folded SCR3, like SCR1-3, fluoresces with an intensity 13.7-fold lower than when unfolded.

Kinetics of SCR3 unfolding and folding.

Stopped-flow fluorescence studies were again used to follow the kinetics of unfolding of SCR3 in various concentrations of GdnHCl or the refolding of SCR on dilution from 7 M GdnHCl. These data were found to be very different from those for the parallel processes in SCR1-3 (see Figure 6); both folding and unfolding processes are single-exponential events and as such probably describe single folding and unfolding events.

Figure 6 shows that there appears to be no curvature in the plot of lnK of folding against GdnHCl concentration, suggesting that the rate of folding at low concentrations of denaturant is not rate limited by another process. Furthermore, the midpoint of the unfolding curve seen in Figure 4 (2.2 M GdnHCl) is virtually identical with the value where the rates of folding and unfolding are at a minimum. Figure 6 also shows that at all temperatures employed, the rates of unfolding of SCR3 are faster and the rates of refolding slower than those for SCR1-3.

Analysis of the effect of temperature on the rate of unfolding showed that ΔHf for SCR3 is 60.4 kJ/mol. Interestingly, the temperature resulting in the apparent maximum rate of refolding of SCR3 is 32.8°C (see Figure 8) which is substantially higher than that for SCR1-3.
Discussion

We have presented studies on the conformational stability of SCR1–3, SCR3 and the unfolding and folding processes initiated by the addition or removal of the denaturant GdnHCl. In SCR1–3, one conserved Trp residue is located in each domain at positions 51, 113 and 184, respectively. A fourth, non-conserved, Trp residue is located at position 7 close to the N-terminus of the first domain. In SCR3 the sole Trp residue is equivalent to residue 184 in SCR1–3. Each of the conserved residues is equivalent to the Trp residue found at position 52 of the sixteenth repeat from Factor H (Norman et al., 1991). The fluorescence emission spectrum of the protein has a maximum at 338 nm, typical of emission from Trp residues which are partially exposed to solvent. The intensity of fluorescence emission of the folded state is increased approximately twofold by the reduction of the disulphide bridges but is greatly increased (13.7-fold) upon denaturation with GdnHCl with a new emission maximum at 348 nm. The increase in fluorescence intensity suggests that the Trp residues are subject to static quenching in the folded state and that this is diminished by the release of folding constraints on addition of denaturant. Aromatic side chains are known to form specific non-covalent bonds of considerable strength with disulphides (Morgan et al., 1978; Nemethy and Scheraga, 1981). The interaction takes place between the sulphur atoms and the electron system of aromatic rings and could play a part in the high stability of SCR1–3. Molecular graphical modelling of a single domain of SCR1–3 onto the sixteenth domain of Factor H indicates that the tryptophan residue found in each domain is located in close proximity to the N-terminal disulphide bond (see Figure 1), possibly allowing this interaction to take place. Cowgill (1967) demonstrated that a disulphide–tyrosine interaction in ribonuclease A quenched the fluorescence of the tyrosine involved. It is therefore plausible that when the disulphide bonds in SCR1–3 are reduced, the tryptophan residues are released from this interaction and so the quantum yield of fluorescence is increased (no change in absorption takes place). It is of interest that the fluorescence characteristics of SCR3 appear identical with those of SCR1–3, despite the latter containing one non-conserved Trp residue in SCR1 in addition to the conserved Trp in each domain, suggesting that the fluorescence intensity of the former is very low or that it changes little on unfolding.

The significant fluorescence and CD signal changes between native and denatured SCR1–3 allowed unfolding to be monitored. The conformational stability of 19.6 kJ/mol at 25°C for non-reduced SCR1–3 was determined from three different techniques (fluorescence and far- and near-UV CD). These gave similar results for all three parameters measured (\(G_{H2O}\), m and mid-point of denaturation), suggesting that the same unfolding processes and conformational stability are being measured. The transition phases of the unfolding curves indicated a single unfolding process for both SCR3 and SCR1–3 giving a linear curve (inset to Figure 4). Thus it would appear that all three domains unfold with the same dependence upon the concentration of denaturant. The considerable reduction in \(G_{H2O}\) (−10 kJ/mol) in the presence of 50 mM β-mercaptoethanol is evidence for the significant contribution of the disulphide bonds to the stability of the protein.

There is the possibility, however, that the three domains unfold individually with similar, but not identical, mid-points of the transition. Unfolding intermediates such as one domain unfolded but the other two remaining folded may not be detected by equilibrium studies. The overall unfolding process would give rise to a shallower, less cooperative transition with a lower value of m. We believe that this possibility can be excluded since the kinetic studies indicate that the major part of the fluorescence change occurs at one rate and that the midpoint of the transition determined by equilibrium studies agrees with that value where the rates of folding and unfolding are at a minimum. This discussion is clarified by the unfolding characteristics of SCR3. The identical CD spectra of SCR3 and SCR1–3 in the far-UV region and the identical transitions in fluorescence spectra on denaturation suggest that domains in both native proteins are similarly folded. However, the data in Figures 4 and 6 show that SCR3 is much less stable (conformational stability = 12–13 kJ/mol at 25°C) than when in the three domain construct. The decreased stability arises from an approximately fourfold increase in the rate of unfolding of SCR3 due to a significantly reduced activation enthalpy of unfolding of 60.4 kJ/mol compared with 119 kJ/mol for SCR1–3. The stability is further decreased by an approximately twofold lower rate of refolding (at 25°C).

The temperature dependence of denaturation in the presence of GdnHCl allowed an estimate of the change in heat capacity of unfolding to be calculated for SCR1–3 of 6.6 kJ/K.mol or 33.8 J/K.mol amino acid. The positive \(C_p\) observed upon protein unfolding is thought to result mainly from the exposure of buried non-polar side chains to solvent (Baldwin, 1986). The measured values for the heat capacity change for the unfolding of simple proteins with a single polypeptide chain are 55 ± 10 J/K.mol of amino acid, regardless of the perturbation used to shift the equilibrium (Pace and Tanford, 1968). It has been noted, however, that changes in heat capacity of unfolding decrease as the number of participating disulphide bonds in the polypeptide increases (Doig and Williams, 1991). SCR1–3 has six disulphide bonds and so this may well explain its lower than average heat capacity. The low \(C_p\) between unfolded and folded states of such proteins is probably due to the disulphide bonds reducing the hydrophobic effect, defined as the process by which non-polar groups are removed from contact with water (Doig and Williams, 1991), rather than being due to positive changes in the heat capacity directly due to the presence of disulphide bridges (Kyte, 1995).

The kinetics of folding and unfolding were investigated on non-reduced SCR1–3 and SCR3. Such transitions do not represent full folding/unfolding processes because in the unfolded state the presence of correctly paired disulphide bonds brings the cross-linked random coil closer to the native structure both thermodynamically and kinetically. Nevertheless, important insights into the kinetics of folding can be gained. Kinetic experiments enable the detection of intermediates which are often not detected under equilibrium conditions. Although the equilibrium properties of SCR1–3 and SCR3 and the kinetic properties of SCR3 suggest a two-state transition, the kinetic properties determined for SCR1–3 clearly indicate the presence of intermediates.

The rate constants of unfolding and refolding for SCR3 and SCR1–3 are sensitive to the denaturant concentration. The higher the denaturant concentration, the faster both proteins unfold and the slower they refold. In the case of SCR1–3, both unfolding and refolding appear to be biphasic processes describable by two rate constants. However, the slower rate of fluorescence change noted for the unfolding process is questionable due to unfolding since it alone appears to be
independent of denaturant concentration and too fast for cis–trans isomerizations around prolyl residues. It may therefore arise from other spectral perturbations. The faster phase shows a dependence upon GdnHCl concentration which results in a linear plot of lnk against GdnHCl concentration for unfolding. The activation enthalpy for the unfolding of SCR1–3 in the absence of denaturant is 119 KJ/mol and is considerably higher than that for SCR3 (60.4 KJ/mol) or the average value of 51 KJ/mol for chemical processes (Price and Dwek, 1986), suggesting that a major conformational change occurs. The lower value for SCR3 is consistent with more rapid unfolding kinetics and its decreased stability.

Experiments carried out on the single-domain SCR3 shows that both the unfolding and refolding are monophasic, suggesting that the slower rate of the two rates noted in the refolding of SCR1–3 (see Table II) may be due to inter-domain interactions that cannot occur in a single-domain construct. Nevertheless, the possibility that the slower rate of fluorescence change seen in refolding studies of SCR1–3 is due to spectral perturbations of the non-conserved Trp7, cannot yet be excluded.

Both phases of the refolding process of SCR1–3 are dependent upon denaturant concentration and each offers a significant contribution to the overall fluorescence change. Data in Figure 6 shows that a plot of the natural logarithm of the fast rate constant for the SCR1–3 folding process against GdnHCl concentration becomes non-linear below 2 M GdnHCl. This deviation from linearity is strong evidence for the existence of kinetic folding intermediates that become rate limiting in conditions strongly favouring folding (Matouschek et al., 1990). In contrast, the data in Figure 6 show that SCR3 appears to fold in a simple process.

The rates of refolding of SCR1–3 and SCR3 in 0.63 M GdnHCl were investigated over a range of temperatures. The rate constants for folding were found to increase initially with temperature, plateau and then decrease. This decrease in the rate constant with increasing temperature results in an apparently negative value for the enthalpy of activation for refolding of SCR1–3 and SCR3. Oliveberg et al. (1995) have already demonstrated that this phenomenon occurs in the refolding of barnase and chymotrypsin inhibitor 2, and have suggested that the activation enthalpy of refolding is dependent upon temperature owing to the large change in the specific heat capacity. The negative enthalpies could be the result of changes in the heat capacity of the system due to the change in exposure of hydrophobic surfaces to water as the structure of the unfolded protein approaches that of the transition state, although other possibilities exist (Oliveberg et al., 1995).

In summary, fluorescence and CD spectroscopic techniques have been used to follow the folding and unfolding transitions and overall conformation of SCR1–3 and SCR3 of CR1. We have shown that SCR1–3 is a remarkably stable protein and we speculate that this stability may be due, in part, to an interaction between the conserved tryptophan residue and one of the disulphide bonds in each SCR domain. In addition, we have demonstrated that folding is a biphasic process and possibly occurs through an intermediate species. The observation that the major part, if not all, of the change in fluorescence signal associated with unfolding is described by a single rate constant cannot be used to distinguish between two possibilities: first, that the three domains change structure independently but at the same rate, or second, that SCR1–3 unfolds as a single cooperative unit. However, comparison of the kinetic data shows that the unfolding of SCR1–3 is a much slower process than that of SCR3. No evidence of a process at a rate attributable to the unfolding of SCR3 could be found in the unfolding of SCR1–3, suggesting that SCR1–3 unfolds as a single unit. Other studies show that SCR3 refolds at a much slower rate in isolation than when in SCR1–3, again suggesting that the latter refolds as a unit and that some interactions may exist between domains. Ongoing structural studies should help clarify the extent of domain interactions and whether such interactions are similar to those described by Barlow et al. (1993) for Factor H15–16.

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References


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