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# Microsecond structural fluctuations in denatured cytochrome c and the mechanism of rapid chain contraction

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**Abstract**

In order to improve the understanding of the diffusive chain movements leading to protein folding, we have studied microsecond conformational fluctuations in denatured yeast cytochrome c by fluorescence correlation spectroscopy (FCS). We show that emitted fluorescence from the dye Alexa-488 chemically attached to the protein depends on the extension of the chain, such that fluctuations in chain length will give fluctuations in fluorescence intensity. Exposure to chemical denaturants leads to an increase in diffusion times, indicating expansion of the molecule. Structural fluctuations of the unfolded protein give rise to fluctuations in the emitted fluorescence. However, FCS measurements fail to show conformational fluctuations of chain segments, establishing an upper bound of 4 $\mu$s on the timescale of chain fluctuations in the denatured state. This clearly shows that an early process with a time constant of 50 $\mu$s observed in folding experiments must involve passage of a free energy barrier, and cannot be barrierless chain collapse as has been proposed.

(Some figures in this article are in colour only in the electronic version)

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## 1. Introduction

Protein folding and unfolding dynamics cover a wide range of timescales (Thirumalai 1995). Motions of amino acid side chains and the folding and unfolding of short peptide segments (motifs) occur in the picosecond and nanosecond time range (Phillips *et al* 1995), and secondary structures can be formed in the nanosecond to microsecond timescale. In the last few years, submillisecond folding kinetics has been measured for a few proteins, in particular equine cytochrome c (Chan *et al* 1997, Shastry and Roder 1998, Yeh and Rousseau 1998, Pollack *et al* 1999). The spectroscopic signals change in at least two phases, one with a lifetime of $\sim$50 $\mu$s and one with a lifetime of $\sim$600 $\mu$s. It has been discussed (Yeh and Rousseau 2000) whether the fast process should be interpreted as an activated process (Shastry and Roder 1998) or as barrierless chain compaction (Chan *et al* 1997, Sosnick *et al* 1997). Barrierless chain compaction is understood as the immediate response to an induced shift in the most favourable radius of gyration. Therefore, it must occur at the timescale of structural fluctuations in the unfolded state. In the present work, we use fluorescence correlation spectroscopy (FCS) to study the timescale of such fluctuations in denatured cytochrome c, in order to determine whether chain compaction is rate limiting in the initial folding event.

Only a few quantitative measurements of fluctuations in denatured proteins have been performed so far. In flash photolysis experiments, Eaton and co-workers (Jones *et al* 1993, Hagen *et al* 1996) found the time for met–haem contact formation in denatured cytochrome c to be $\sim$40 $\mu$s. Modelling of time-resolved fluorescence resonance energy transfer (FRET) data (Gottfried and Haas 1992, Lakowicz *et al* 1993) gave intrachain diffusion constants that for a 25-residue stretch in troponin I translate into a correlation time of about 0.2 $\mu$s (Lakowicz *et al* 1993). Hagen *et al* (1996) calculated similar intrachain diffusion constants from the met–haem contact times.

Figure 1 shows the experimental principle we applied. An FCS instrument measures fluorescence from a small confocal volume typically on the order of 1 fl (1 $\mu m^3$) inside the liquid sample, and quantifies the timescales of intensity fluctuations. These fluctuations can arise from the diffusion of proteins into and out of the confocal volume, but also from internal processes that give rise to variations of fluorescence of the fluorescence tag (probe) on the protein. Chattopadhyay *et al* (2002) detected a 35 $\mu$s motion in folded intestinal fatty acid binding protein in this way, with the variation in fluorescence intensity arising from fluorophore binding in a pocket on the protein. Here we show that cytochrome c isozyme-1 from yeast can be labelled with a fluorescent dye in such a way that the fluorescence depends on the conformational state of the protein. Structural fluctuations of the unfolded protein will therefore give rise to fluctuations in the emitted fluorescence. The dependence of fluorescence intensity on conformation is due to distance-dependent FRET from the dye (donor) attached close to the C terminus, to the endogenous, non-fluorescent haem group (acceptor) that in cytochrome c is covalently linked to the peptide chain close to the N terminus. Previously, Deniz *et al* (2000) have studied single-molecule FRET without time resolution with chymotrypsin inhibitor 2. Particular properties of this protein allowed them to incorporate both a fluorescent acceptor and a fluorescent donor. Schuler *et al* (2002) performed a similar experiment on the heat-shock protein Csp*Tm*. The experiments of our study are most sensitive in the time range from a few microseconds (Kim *et al* 2002) up to the time for diffusion through the confocal volume, which for cytochrome c is on the order of a few hundred microseconds. Because the overall folding process itself is much slower (Sosnick *et al* 1994, Yeh and Rousseau 1998), only motion within the ensemble of denatured states can be detected. Kim *et al* (2002) used the same technique on single immobilized RNA molecules to detect millisecond conformational changes, and Wallace *et al* (2000) a related method to study conformational dynamics of a DNA hairpin loop on timescales from 10 $\mu$s to 1 ms.

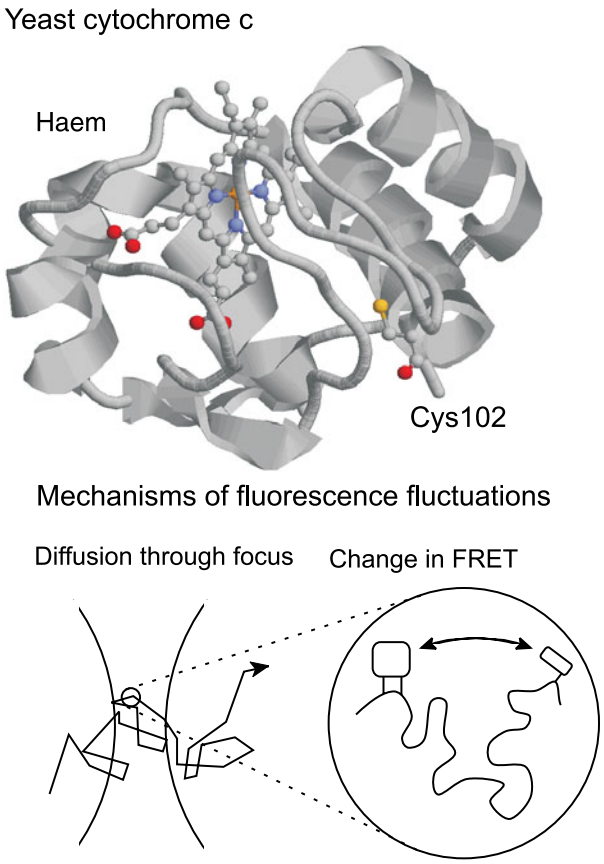


**Figure 1.** Principle of the experiments. The protein is labelled with a fluorescent dye at Cys-102 in such a way that the fluorescence depends on the extension of the peptide chain by virtue of FRET to the haem, covalently linked to Cys-14 and Cys-17. FCS quantifies fluctuations in fluorescence intensity from the fluorescently labelled protein. Diffusion through the confocal volume gives rise to fluctuations on the timescale of a few hundred microseconds, and changes in the donor–acceptor distance on faster timescales will give rise to additional contributions.

## 2. Materials and methods

Cytochrome c from *Saccharomyces cerevisiae* was purchased from Sigma–Aldrich as a mixture of isoform 1 (mainly) and 2. Isoform 1 was purified using the procedure of Satterlee *et al* (1988). Mass spectroscopy and N-terminal sequencing further confirmed the identity of the product. The fluorescent dye Alexa-488 maleimide (Molecular Probes) was conjugated to the protein under anaerobic conditions as follows. After purification, the protein was in a 1.5 mg $ml^{-1}$ buffer solution (50 mM $Na_2HPO_4$, pH 7.0). The single free cysteine residue was reduced by adding 30 $\mu$l $ml^{-1}$ of mercaptoethanol, incubating for 15 min and passing the solution over a prepacked G-25 column (NAP10, Pharmacia Biotech AB, Sweden) equilibrated with 4 M GuHCl, 50 mM $Na_2HPO_4$, pH 7.0. The elution volume was 1 ml. 1 mg fluorescent dye was dissolved in 174 $\mu$M DMF, mixed with the protein solution and incubated for 4 h in the dark. Excess dye was removed by gel filtration on another NAP10 column.

Digestion of the modified protein was carried out with an Asp/Glu specific endoproteinase from *Staphylococcus aureus* V8, kindly supplied by Novozymes, Bagsværd, Denmark.

To ensure complete digestion, the protein solution was incubated with 50 $\mu$g ml$^{-1}$ protease overnight at 37 °C. All measurements were performed in 50 mM $Na_2HPO_4$, pH 7.0 at 20 °C. Absorption spectra were measured on an HP 8453 UV–visible spectrophotometer. Concentrations were determined by using extinction coefficients of 96 mM$^{-1}$ cm$^{-1}$ at the Soret maximum for cytochrome c and 72 mM$^{-1}$ cm$^{-1}$ for Alexa-488. Fluorescence spectra were measured on an LS-55 Perkin-Elmer luminescence spectrometer and fluorescence intensities were calculated by integrating from 505 to 525 nm. The equilibrium denaturation curves were fitted by the function (Fersht 1999)

$$\text{fluorescence} = \frac{\alpha_N + (\alpha_D + \beta_D[\text{denaturant}])\exp(\{m[\text{denaturant}] - \Delta G^{H_2O}\}/RT)}{1 + \exp(\{m[\text{denaturant}] - \Delta G^{H_2O}\}/RT)}$$

where $\alpha_N$, $\alpha_D$, $\beta_D$, $m$ and $\Delta G^{H_2O}$ are free parameters. Since the properties of unfolding are poorly determined in the GuHCl denaturation, the value of $\Delta G^{H_2O}$ found from the urea denaturation data (1.5 kcal mol$^{-1}$) was used as a fixed parameter in fitting the GuHCl-denaturation data.

The dye–haem Förster distance $R_0$ was calculated from standard expressions (Förster 1948), using the value of two-thirds for the orientational factor $\kappa^2$. We did not perform measurements of the quantum yield of Alexa-488, but according to the manufacturer the dye is very bright. We have taken a value of 0.5 for which the Förster distance is $R_0 = 43$ Å; values of 0.25 and 0.75 give $R_0 = 39$ and 46 Å, respectively. The average haem–dye distance $r$ can be calculated from the ratio $\alpha$ of the fluorescence in the presence of haem to the fluorescence in the absence of haem (the digest) as

$$r = R_0 \sqrt[6]{\alpha/(1-\alpha)}. \tag{1}$$

When the sample molecules have a distribution of different distances, the large distances are weighted more, and so the expression tends to give too high values.

FCS measurements were done on a Confocor 1 (prototype, Zeiss/Evotech, Germany) with a Zeiss Neofluar 40× NA 1.2 water immersion objective. The sample was excited at 488 nm and fluorescence detected through a 505–545 nm bandpass filter. Samples of approximately 30 $\mu$l volume were applied to the bottom of an eight-well Nunc plate and measurements of the fluorescence autocorrelation function started immediately. The confocal volume was placed 180 $\mu$m above the glass–water interface. Due to adsorption of protein at the cover glass surface, the signal intensity was only stable for the first 60 s, so only data from this period were collected for analysis.

The intensity autocorrelation function has the property that independent sources of fluctuations give rise to independent factors in the expressions. If, as is usual, the fluctuations are due to diffusion and to triplet state dynamics of the dye, the autocorrelation function is given (Aragon and Pecora 1976, Rigler *et al* 1993)

$$g_D(t) = 1 + \frac{1}{N}\frac{(1 - T + T\exp(-t/\tau_t))}{(1 + 4Dt/w_{xy}^2)\sqrt{1 + 4Dt/w_z^2}} \tag{2}$$

where $N$ is the average number of molecules in the confocal volume, $T$ is the amplitude of the triplet state component, $\tau_t$ is the triplet state lifetime, $D$ is the diffusion coefficient and $w_{xy}$ and $w_z$ are the axes of the ellipsoidal confocal volume. The ratio $w_z/w_{xy}$, which is a property of the apparatus, was in all data fitting held at the constant value of seven found in measurements on pure dye solutions. Equation (2) is exact when both laser focus and point spread function are Gaussian; this is in fact not the case for the point spread function, but (2) has been shown to be a very good approximation (Rigler *et al* 1993).

We have modelled the fluctuations due to changes of the donor–acceptor distance by an autocorrelation function of the form $1 + A\exp(-t/\tau_f)$, where $A$ is an amplitude and $\tau_f$ the

timescale of fluctuations. Such an autocorrelation function arises both in the case of random jumps between two states with different intensity and in the case of diffusion within a Gaussian distribution of intensities. The model autocorrelation function for the labelled protein then becomes

$$g_f(t) = 1 + \frac{1}{N}\frac{(1 - T + T\exp(-t/\tau_t))(1 + A\exp(-t/\tau_f))}{(1 + 4Dt/w_{xy}^2)\sqrt{1 + 4Dt/w_z^2}}. \tag{3}$$

It is assumed that under given conditions the dependence of the fluorescence intensity on donor–acceptor distance can be approximated by the first order expansion around the average value $r_0$:

$$I(r) = I_0 + I'(r - r_0). \tag{4}$$

The fluctuation amplitude $A$ is then given by $A = (\Delta r I'/I_0)^2$, where $\Delta r$ is the standard deviation of the donor–acceptor distance distribution. $I_0$ is known from the ensemble measurements and $I'$ can be obtained from the Förster theory, so both $\Delta r$ and $\tau_f$ can, in principle, be obtained from the fluorescence intensity autocorrelation function.

Since small amounts of unreacted dye are often present in the solution of labelled proteins, we in each case performed a measurement on free dye under the same solution and fitted the data to equation (2). Thus, the total function used for the analysis of the protein samples was

$$f(t) = g_f(t) + \beta(g_D(t) - 1) \tag{5}$$

where the parameters in the dye autocorrelation function $g_D$ were held fixed at the values obtained for the free dye, and $\beta$ as well as the parameters in $g_f$ were allowed to vary.

Data analysis was done using the R software (http://www.r-project.org). Data uncertainties were calculated from Poisson (counting) statistics.

## 3. Results

Cytochrome c isozyme-1 from yeast has 108 amino acids, a haem group covalently linked to cysteines in the 19th and 22nd positions from the N terminal (conventionally numbered 14 and 17) and a free cysteine amenable to chemical modification next to the C terminus (conventionally numbered 102). Figure 2 shows the absorption spectra of modified and unmodified protein, together with the fluorescence spectrum of a sample where the modified protein had been cleaved to several smaller peptides by an Asp/Glu-specific protease. From the absorption spectrum of the modified protein and the extinction coefficients cited in section 2, we derive a dye/protein ratio of 0.99, strongly indicating complete and specific labelling.

The absorption of the unmodified protein at wavelengths above 350 nm is solely due to the haem group. It can be seen from figure 2 that the band around 525 nm overlaps nicely with the emission spectrum of Alexa-488, leading to FRET with a Förster distance of 43 Å. In comparison, the distance between the haem Fe atom and the $C_\alpha$ atom of Cys-102 is 14 Å in the x-ray structure (Louie and Brayer 1990). Figure 3 shows how the fluorescence of the modified protein excited at 488 nm changes on increasing concentrations of urea or GuHCl. The increase in fluorescence reflects an increase in the distance between the haem group and the dye. The full lines through the circles are two-state fits (see section 2). For the GuHCl-denaturation data the curve is fitted to all data points but for the urea-denaturation data only the region 0–5 M is used. The fits give values of $\Delta G^{H_2O} = 1.5$ kcal mol$^{-1}$, $m = 3.3$ kcal mol$^{-1}$ M$^{-1}$ for GuHCl denaturation and $m = 1.0$ kcal mol$^{-1}$ M$^{-1}$ for urea denaturation. Also shown is the fluorescence of the proteolytic digest and an interpolating straight line.

Figure 4 shows the diffusion time $\tau$ for Alexa-488 labelled cytochrome c as function of denaturant concentration. The times have been corrected for the increase in viscosity with

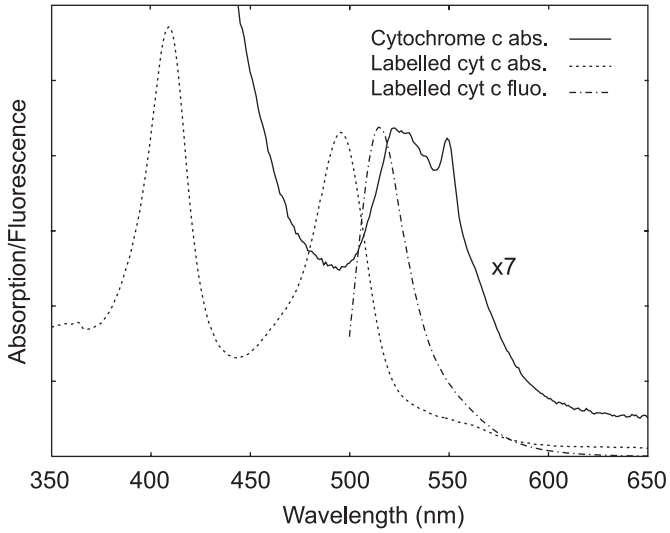


**Figure 2.** Absorption spectra of the modified protein, unmodified protein (×7) and fluorescence spectrum of digested protein.

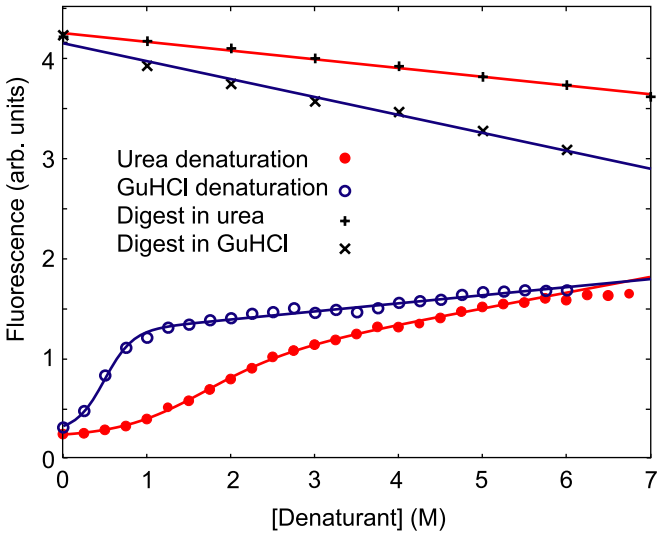


**Figure 3.** Dependence of fluorescence from the labelled protein and proteolytic digests on denaturant concentration.

increasing concentration of denaturant, by taking the diffusion time of the free dye under given conditions to be proportional to the viscosity. The corrected diffusion time is seen to increase from about 140 $\mu$s in the native state to about 220 $\mu$s for the denatured protein. The diffusion time $\tau_{diff}$ is related to the diffusion coefficient $D$ and the width $w_{xy}$ of the confocal volume in the $xy$-plane by $\tau_{diff} = w_{xy}^2/4D$, so the results indicate a decrease in diffusion coefficient of about 35% upon denaturation.

It is clear from the comparison of intact and cleaved protein shown in figure 3 that significant quenching by FRET occurs in the denatured state. Fluctuations of the donor–

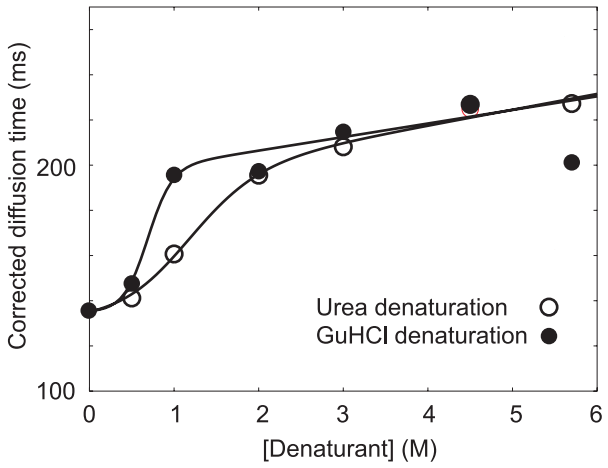


**Figure 4.** Diffusion times of fluorescently labelled cytochrome c as function of denaturant concentration, corrected to the viscosity at zero denaturant concentration. The solid curves are guides to the eye.

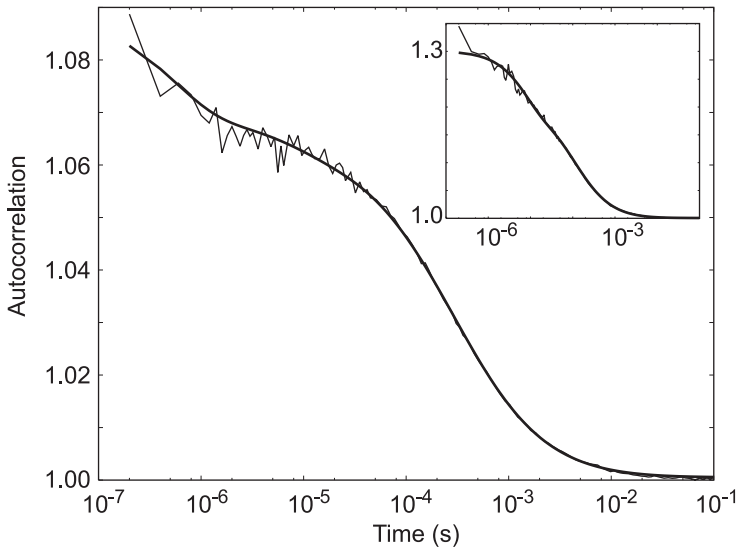


**Figure 5.** Fluorescence intensity autocorrelation function of labelled protein in 4.5 M urea, pH 7.0. Inset: autocorrelation of Alexa-488 dye under the same conditions. The bold curves are fits described in the text.

acceptor distance in the denatured protein should therefore give rise to fluctuations in the fluorescence intensity. As noted in the introduction, parts of these structural fluctuations are expected to be in the low end of the time window accessible to FCS. However, no significant fluctuations could be seen in our data on this timescale. Figure 5 shows the autocorrelation function recorded at 4.5 M urea. The solid curve is a fit to a model without structural fluctuations ($A = 0$ in formula (3)), and this is seen to give a satisfactory description of the data ($\chi^2 = 1.33$, while $\chi^2 = 1.29$ for the fit to the free dye data). Similar results were obtained at all conditions studied.

## 4. Discussion

The equilibrium denaturation data in figure 3 are well described by a two-state model with linear dependence of the fluorescence in the denatured state on denaturant concentration, except for the urea-denaturation data above 5 M concentration. In the digestion experiments with the Glu/Asp-specific protease, we used high protease concentration in order to get cleavage at all Glu and Asp residues. In this case, the dye labelled cysteine is located in a peptide fragment without the haem, and at the concentrations used in our experiments ($<10\ \mu$M) the fluorescence is unaffected by FRET. Under these conditions, the fluorescence is seen to decrease with increasing denaturant concentration. The steady increase in fluorescence seen for the intact protein therefore implies a decrease of FRET, due to an increase of the average donor–acceptor distance. This has previously been observed for cytochrome c using tryptophan–haem energy transfer (Tsogn 1976, Jeng and Englander 1991, Chan *et al* 1997). Although most of the data are well described by a linear dependence of the denatured state fluorescence on denaturant concentration, this fit is in fact purely empirical, and the deviation observed above 5 M urea does not necessarily imply a second transition. From equation (1) the haem–dye distance in the denatured state can be calculated at values around 40 Å, depending on the conditions.

The folded state is significantly destabilized by the attachment of the dye: the free energy of unfolding calculated from the urea denaturation data is 1.5 kcal mol$^{-1}$, compared to 5.8 for the unmodified protein (Mines *et al* 1996). It is seen from figure 1 that the thiol group of Cys-102 points to the interior of the unmodified protein; the distortion necessary for labelling with the relatively bulky dye probably explains the destabilization. We do not expect the unfolded state to be significantly affected.

The diffusion times plotted in figure 4 show that the diffusion coefficient, which is inversely proportional to the diffusion time, decreases by about 35% upon denaturation of yeast cytochrome c. Segel *et al* (1998) measured the [GuHCl] dependence of the radius of gyration for equine cytochrome c using small angle x-ray scattering, and found an increase of about 130% upon unfolding at pH 7. Hagihara *et al* (1998) found the radius of gyration to increase by 92% at pH 3.2. For a sphere, the diffusion coefficient and the radius of gyration $R_G$ are related (Cantor and Schimmel 1980)

$$D_S = \frac{kT}{24.3\eta R_G}$$

where $k$ is Boltzmann's constant, $T$ the temperature and $\eta$ the viscosity, while for a random coil the relation is (Cantor and Schimmel 1980):

$$D_{RC} = \frac{kT}{12.5\eta R_G}.$$

If denaturation is modelled as a transition from a compact sphere to a random coil, the measurements of Segel *et al* (1998) would predict a decrease of 15% in the diffusion coefficient (still corrected for the change of viscosity). Assuming that our data and those of Segel *et al* measure almost identical states, the larger decrease in $D$ that we observe indicate that the denatured state diffuses more slowly than a random coil of the same radius of gyration, probably due to a less open structure.

The presence of a fraction of denatured protein even at 0 M denaturant could potentially skew the measurement, in particular since the fluorescence from the denatured protein is much more intense than that of the native protein. We checked this by performing the measurement on protein labelled with tetramethylrhodamine, which has much less contrast between native and denatured states, and obtained identical results (not shown).

The process of protein folding/unfolding is not a local transition, but rather involves diffusive motion of the entire chain. Here, we have aimed at measuring the amplitude of such

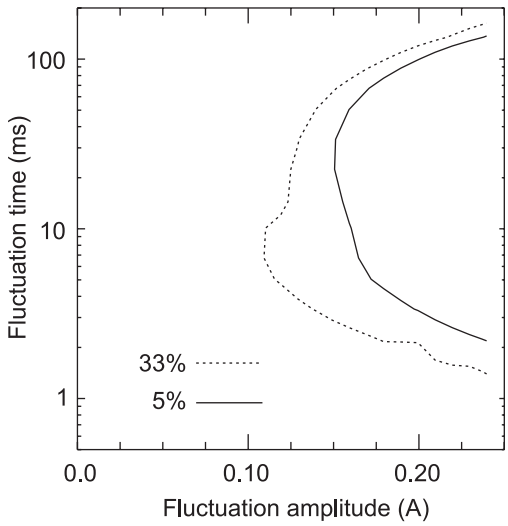


**Figure 6.** Upper limits on microsecond fluorescence fluctuations in 4.5 M urea. For each point in the plot, the data were fitted with fixed amplitude and correlation time of the hypothetical fluctuation as described in the text, and the probability of having a fit of this quality for a correct model was calculated. The 33 and 5% probability contour lines are shown.

motions in the denatured state on the microsecond timescale. However, the data and fits in figure 5 show that data obtained in 4.5 M urea can be fitted to a model without any structural fluctuations at all, virtually as well as the free dye data can be fitted to equation (2) (inset). The same conclusion was reached in all other conditions studied. Since the denatured chain must in fact undergo *some* fluctuations, we investigated to what extent the data could give upper limits on the amplitude of the motions. The measured autocorrelation functions were fitted to equation (5) with $A$ and $\tau_f$ held at different, *fixed* values. We found the ($A$, $\tau_f$) values for which the relative increase in $\chi^2$ was $\sqrt{2/M}$ and $2\sqrt{2/M}$, where $M$ is the number of data points used from the autocorrelation function ($M = 108$). These increases in $\chi^2$ would roughly occur by chance with probabilities of 33 and 5%, respectively. The corresponding contours in the ($A$, $\tau_f$)-plane obtained at 4.5 M urea are shown in figure 6. The figure shows that the experiment is most sensitive to fluctuation times in the range 4–70 $\mu$s, where it places an upper bound on the amplitude at about 0.16 ($\chi^2$ increased by $2\sqrt{2/M}$). *If* the main structural fluctuations were on this timescale, the standard deviation of the donor–acceptor distance distribution should be $\Delta r \leqslant 4$ Å. Donor–acceptor distance distributions measured on denatured proteins typically show standard deviations several times larger (Lakowicz *et al* 1994, Ittah and Haas 1995). The most likely explanation of our result is that the fluctuations are in fact on a timescale $\leqslant 4$ $\mu$s. Here, the experiment is much less sensitive, since the effects of structural fluctuations are indistinguishable from triplet state effects. We note that another reason for the absence of observable movements could be ligation of His or Met residues to the haem group in the denatured protein (Sosnick *et al* 1994, Hammack *et al* 1998, Hagen *et al* 2002), which would constrain the chain geometry. However, measurements with and without 50 mM imidazole gave identical results for equilibrium fluorescence as well as for FCS, ruling out this possibility (results not shown).

Our results are obtained for yeast cytochrome c, but it is highly plausible that denatured equine cytochrome c would behave in much the same way. The data show that the 50 $\mu$s process of chain compaction (Shastry and Roder 1998, Pollack *et al* 1999) is more than tenfold slower

than the timescale of fluctuations in the denatured state, strongly supporting the view that some type of free energy barrier passage is involved (Shastry and Roder 1998). Circular dichroism data indicate that about 20% of native $\alpha$-helix content is formed in the rapid phase (Chen *et al* 1999, Akiyama *et al* 2000) and the rate is quite temperature dependent, corresponding to an activation enthalpy of 7.5 kcal mol$^{-1}$ (Shastry and Roder 1998). The hypothesis that passage through the transition state involves liberation of bound water molecules in order to permit internal contacts to form is consistent with these results.

In transition state theory, the rate of barrier passage is given by

$$k_f = k_0 \exp(-\Delta G/k_B T)$$

where $\Delta G$ is the free energy difference between the denatured state and the transition state; $k_B$ is Boltzmann's constant and $T$ the absolute temperature. The prefactor $k_0$ depends on how fast the protein structure fluctuates around the top of the barrier; it essentially measures how rapidly the protein leaves the transition state (Risken 1996). Folding experiments usually only deal with relative changes of the folding rate, interpreted as due to changes of $\Delta G$; $k_0$ is considered an unknown constant. Recently, it was pointed out that polymer theory and experiments on denatured proteins can provide rough limits on the *absolute* value of $k_0$, and thereby an estimate on the absolute value of $\Delta G$ (Schuler *et al* 2002). These authors obtained an upper limit of 25 $\mu$s for the polypeptide reconfiguration time in the unfolded state of the cold-shock protein Csp*Tm*. Our FCS data improve this bound by almost an order of magnitude. The main folding event in cytochrome c can happen in as little as 600 $\mu$s (Chan *et al* 1997). The motions necessary to take a protein through the main transition state for folding are not known, but again the end-to-end fluctuation time of the unfolded chain appears to be a safe upper estimate. Setting $k_f > 600$ $\mu$s and $k_0 < 4$ $\mu$s one obtains $\Delta G/kT > 3$ kcal mol$^{-1}$. Shastry and Roder (1998) obtained an enthalpic contribution to the barrier $\Delta H$ of only $\sim$1 kcal mol$^{-1}$, which indicates that the barrier has a large entropic contribution.

## 5. Conclusion

We have developed a model system that allows cytochrome c folding to be studied with single-molecule methods. Diffusion measurements with FCS show that the hydrodynamic radius of the protein expands by about 35% upon denaturation. Analysis of the data provides an upper limit to the end-to-end fluctuation time of about 4 $\mu$s. This supports the view that the initial process with a lifetime of 50 $\mu$s observed in cytochrome c folding is slowed down by passage of a free energy barrier, and so is not barrierless. Comparison with known activation enthalpies also suggests that the barrier for folding to the native state is for the most part entropic.

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