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Biophysical Chemistry 101–102 (2002) 155–165

Biophysical Chemistry

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Efficacy of macromolecular crowding in forcing proteins to fold☆

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Received 2 January 2002; accepted 19 February 2002

Abstract

The intrinsically unstructured protein, reduced and carboxyamidated RNase T1 (TCAM) was used to determine the degree to which macromolecular crowding agents increase the equilibrium constant for folding. TCAM is not catalytically active in an aqueous assay system alone, but becomes catalytically active on addition of 400 mg/ml dextran 70. The activity observed accounts for approximately 16% of the total available TCAM in solution. We interpret this result to mean that 16% of the TCAM becomes folded protein in the presence of the 400 mg/ml dextran 70, and this translates into an approximately five-fold increase in the equilibrium constant for folding. Sarcosine-induced folding of TCAM was performed in the presence of 0, 100, 200 and 300 mg/ml dextran 70, and *apparent* ΔG^{o}_{N-D} values determined from the linear extrapolation method provide an estimated 22% folded TCAM formed in the limit of zero sarcosine concentration and in presence of 400 mg/ml dextran 70. The increase in TCAM folding equilibrium constant using this method of determination is approximately 7.5-fold. Overall, the results indicate that macromolecular crowding agents are only modestly effective in promoting folding of this intrinsically unstructured protein.

Keywords: Osmolyte; Macromolecular crowding; Protein stability; Protein folding; Intrinsically unstructured; Excluded volume

1. Introduction

It is well recognized that living cells contain a complex array of large and small molecules, creating a crowded and confining environment in which life-giving processes occur [1–7]. For macromolecular reactions conducted in the presence of high background concentrations of non-reacting macromolecules (macromolecular crowding agents), excluded volume theory predicts that the reaction will be shifted in the direction of those species (either products or reactants) that collectively have the smaller excluded volume [2,7]. In the simplest cases, protein folding involves a monomolecular transition between a compact 'native' globular species and a much less compact 'denatured' species. Based on excluded volume theory, it has been predicted that, in the presence

☆ This paper is dedicated to Prof John Schellman, whose integrity and insightful work over a long and productive career have served as a model to all.

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PII: S0301-4622(02)00148-5

of sufficiently high macromolecular crowding agent, the equilibrium constant for denaturation should decrease by one–two orders of magnitude [3]. Our experimental objective in this report was to assess, for a particular protein, the magnitude of protein stabilization that arises from macromolecular crowding.

Most of the earlier studies on the effects of macromolecular crowding in protein folding have used proteins that have reasonably high stability in aqueous buffer solution [8,9]. The protocol in these studies was to denature the protein in high denaturant concentration, then dilute the protein into zero and higher concentrations of macromolecular crowding agents, such as ficoll 70, dextrans of given molecular weights, and neutral proteins such as BSA, ovalbumin, etc. The results of these studies clearly demonstrate that macromolecular crowding agents greatly affect protein–protein association reactions, and that the kinetics of folding in the presence of these agents can be complex. Although no direct stability measurements were made, van den Berg et al. concluded that crowding does not substantially alter the energetics of the protein folding reaction [9]. Their conclusion that macromolecular crowding effects on protein folding are small was based on the similarity of the accumulation of folding intermediates in the presence and absence of macromolecular crowding agents. In the interest of assessing the thermodynamic stabilization or destabilization effects brought about by macromolecular crowding agents in a more direct manner, it is necessary to select a protein folding system that has appropriate properties. In particular, it is essential that the native $\leftrightarrow$ denatured equilibrium ($N \leftrightarrow D$) under study be reversible, and in order to avoid complications it is highly desirable that the folding process be two-state and devoid of protein–protein associations.

Because of irreversibility and non-equilibrium issues, substantive protein–protein association reactions and the high stability of the native species, the proteins used by van den Berg et al. and Li et al. are not well suited for the purpose of assessing thermodynamic stability effects in the presence of macromolecular crowding agents. The most favorable situation for evaluating protein stability effects is to select a protein that is unstable in aqueous solution and then attempt to force the protein to fold using macromolecular crowding agents. Such was the approach of Flaugh and Lumb, who chose to use such intrinsically unstructured proteins as the kinase-inhibition domain of $p27^{Kip\ 1}$ and the C-terminal domain of c-Fos [10]. However, these authors found no evidence of protein folding in the presence of crowding agents. At 25 °C, Minton's theoretical estimate of the one–two orders-of-magnitude increase in equilibrium constant of protein folding in crowded solutions compared to that in aqueous buffer translates into $\Delta\Delta G^{o}_{N-D}$ of 1.4–2.8 kcal/mol [3]. While this is a significant increase in thermodynamic stability over that of the protein in aqueous solution, if the $D \leftrightarrow N$ equilibrium of the intrinsically unstructured protein lies very much in favor of the intrinsically unstructured (D) form, macromolecular crowding may not be able to shift the equilibrium in favor of the native (N) form sufficiently for the N state to be readily detectable. That is, it may be that folding equilibrium constants for the $p27^{Kip\ 1}$ kinase-inhibition domain and the C-terminal domain of c-Fos favor the unfolded form to such a degree that the one–two order-of-magnitude effect caused by macromolecular crowding is still too small to result in detectable populations of the native species.

In determining the efficacy of macromolecular crowding agents in forcing intrinsically unstructured proteins to fold, we chose to use reduced and carboxyamidated ribonuclease T1 (TCAM), a protein known to be intrinsically unstructured in aqueous buffer solution [11]. We have previously shown that reduction and carboxyamidation of the two disulfide bonds of globular ribonuclease T1 causes the protein to lose its functional activity, as well as its secondary and tertiary structure, and become a highly expanded, denatured ensemble in buffer solution [11]. Addition of naturally occurring osmolytes to this unstructured protein causes it to co-operatively fold, with recovery of its secondary and tertiary structure and significant functional activity. Under the conditions of these studies, it was shown that TCAM in aqueous buffer solution has a ΔG for unfolding of -2.25 kcal/mol. That is, in aqueous buffer solution 98% of

TCAM is in the denatured form and a macromolecular crowding effect of 10-fold or higher on the folding equilibrium constant should produce measurable amounts of the native form of the protein [12]. The data provided in the report herein show a modest, favorable increase in protein stability induced by the macromolecular crowding agent dextran 70 at 400 mg/ml, and a significantly less favorable increase with 400 mg/ml BSA or ficoll 70. The two methods used to evaluate the equilibrium constant for folding of TCAM induced by the macromolecular folding agent, dextran 70, involve detection of catalytically active TCAM and $\Delta\Delta G^{o}_{N-D}$ measurements of the effect of dextran 70 on osmolyte-induced folding of TCAM.

2. Experimental procedures

RNase T1 was expressed and purified by the method of Quaas et al. [13]. Dextran 70, BSA, and iodoacetamide were purchased from Sigma Chemical Co, ficoll 70 and sarcosine were purchased from Fluka, and DTT came from Amresco.

2.1. Preparation of TCAM

RNase T1 lyophilized powder (5 mg) was dissolved in 1 ml of 6 M GdnHCl in 0.5 M Tris buffer containing 1.94 mg reduced DTT, at pH 8.5. After overnight incubation at room temperature, 4.46 mg of iodoacetamide was added with stirring, and the solution was incubated at 25 °C for 15 min. The reaction was stopped by the addition of 10 μl of mercaptoethanol and immersion in ice. The resulting solution was extensively dialyzed against 50 mM Tris/2 mM EDTA, pH 7.5, at 4 °C, and no enzyme activity was observed for TCAM using 2′:3′ cGMP as a substrate. Concentration of TCAM was determined based on Edelhoch's method [14,15]. Concentration of RNase T1 was measured using a molar absorptivity of 19, 215 at 278 nm [16]. The molecular weight of TCAM (11, 320) was verified by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectroscopy, with expected and determined molecular weights always agreeing within 4 Da.

2.2. Fluorescence and CD measurements

All experiments were performed at 22 ± 0.5 °C and pH 7.5. For fluorescence and CD scan spectra, samples of *N*-acetyl-tryptophanamide (NATA) (2.5 μM), TCAM or RNase T1 (10 μg/ml for fluorescence and 0.34 mg/ml for CD) were incubated overnight in 0 or 400 mg/ml crowding agent (dextran 70 or ficoll 70) in 1 mM EDTA and 50 mM Tris, pH 7.5 buffer prior to measurements. Fluorescence emission spectra (278 nm excitation) were collected on a SPEX FluoroMax spectrofluorimeter using 1-cm rectangular cuvettes. Far-UV CD spectra (250–210 nm) were recorded using the CD attachment on an OLIS-RSM spectrometer. Forced folding of TCAM by sarcosine solutions containing fixed concentrations of 0, 100, 200 and 300 mg/ml dextran 70 in 1 mM EDTA, 100 mM Tris buffer was monitored by fluorescence (278 nm excitation and 319 nm emission). Protein aggregation of 30 μg/ml TCAM in 400 mg/ml dextran 70 or ficoll 70 was determined by monitoring 90° light scattered using a SPEX FluoroMax spectrometer at excitation = emission wavelengths of 320, 360, 400, 440 and 480 nm.

2.3. Activity assay

Enzymic activity of folded TCAM and RNase T1 was evaluated by monitoring the loss of substrate (2′:3′cGMP) and appearance of product (3′ GMP) as a function of time. The amounts of substrate and product as a function of time were obtained through integration of the area of their respective ^{31}P NMR peaks determined on a Varian Unity Plus 400-MHz spectrometer. TCAM (30 μg/ml) or RNase T1 (3 μg/ml) was incubated overnight in either 4 M TMAO, 8 M sarcosine, 0 or 400 mg/ml crowding agents (BSA, dextran 70 or ficoll 70) containing 1 mM EDTA and 50 mM Tris buffer, pH 7.5 in 90% water and 10% D_2O. To these solutions was added 20 mM substrate 2′:3′cGMP to start the reaction, monitored by ^{31}P NMR detection. Each reaction was continuously monitored for 6–24 h. Specific activity of RNase T1 or folded TCAM was obtained by evaluating the initial rate of product (3′GMP) formation per mg of protein present.

3. Results

3.1. The issue of crowding-induced aggregation

Because macromolecular crowding is known to promote protein–protein associations, it is essential that a careful examination be made of this issue in the TCAM experimental system. In the presence of macromolecular crowding agents, we sought evidence of TCAM association or aggregation using light scattering and enzyme activity measurements, and through measurements of the reversibility of folding/unfolding of TCAM.

Light scattering, at 90° from incident light, was monitored at fixed (excitation = emission) wavelengths of 480, 440, 400, 360 and 320 nm, using a SPEX FluoroMax spectrofluorimeter. Measurements were conducted upon addition of TCAM [to a final concentration of 30 μg/ml (2.6 μM)] to buffered solutions containing either 400 mg/ml dextran 70 or ficoll 70. The light intensity at 90° from incidence was monitored as a function of time (for 24 h), and no change in light scattering was observed at any wavelength in any of the samples prior to and following addition of the protein (results not shown).

Although buffered solutions of 20 mM 2′:3′ cGMP with and without TCAM showed no hydrolysis of substrate, catalytic activity was detected in the TCAM-containing solution on the addition of crowding agents, such as 300 or 400 mg/ml dextran 70, 400 mg/ml BSA or 400 mg/ml ficoll 70. Conversion of the substrate (20 mM 2′:3′ cGMP) to product (3′GMP) was quantified by integration of the area of the ^{31}P NMR peak for substrate and that for product as a function of time. Fig. 1 shows product vs. time plots in the presence of 400 mg/ml BSA and in 400 mg/ml dextran 70. While linearity is observed in the presence of BSA [or in 400 mg/ml ficoll 70 (not shown) gave a slightly higher rate than did BSA], the slope of the product vs. time plot in the presence of 400 mg/ml dextran 70 is significantly higher and upwardly concave. Because reaction velocity is proportional to the concentration of active enzyme, the increase in TCAM catalytic activity as a function of time is indicative of a small time-dependent increase in functionally active TCAM over the 20+hour time course. Possible reasons for this behavior are deferred to Section 4.

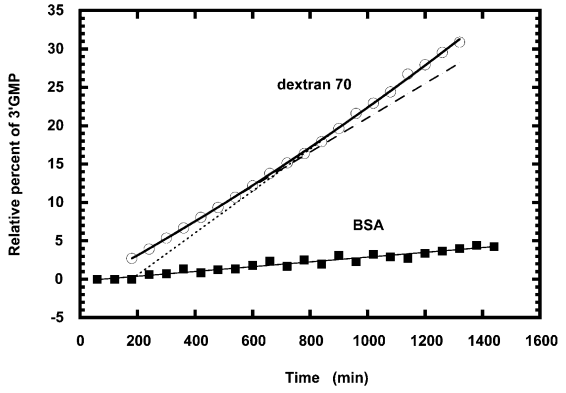

Fig. 1. Activity measurements of TCAM in 400 mg/ml dextran 70 (*open circles*) and in 400 mg/ml BSA (*filled squares*). The relative percentage of 3′GMP is defined as the ratio of the ^{31}P-NMR peak area of product (3′GMP) over the sum of the peak areas of substrate (2′:3′cGMP) and product. The heavy solid line in dextran 70 data is a second-order polynomial fit to the data, and the thin dashed and dotted lines are limiting slopes of the data at 0 and 1300 min, respectively. The thin solid line is the linear fit of the BSA rate data.

3.2. Spectral changes of TCAM in macromolecular crowding solutions

Fig. 2a shows the effects of crowding agents dextran 70 and ficoll 70 on the fluorescence emission spectrum of the tryptophan model compound *N*-acetyl-tryptophanamide (NATA), excited at 278 nm. Ficoll 70 is observed to greatly diminish the fluorescence intensity of the exposed fluorophore without shifting the maximum fluorescence wavelength, while dextran 70 causes a small (~3 nm) blue shift in the wavelength maximum and a 15–20% decrease in fluorescence intensity. The intrinsically unstructured protein TCAM contains a single tryptophan and 9 tyrosine residues. When TCAM is used as the fluorophore (Fig. 2b), ficoll 70 again shows no change in emission maximum wavelength and greatly diminished emission intensity. By contrast, dextran 70 causes a diminished fluorescence intensity of approximately 10%. More importantly, this crowding agent causes a significant blue shift in the

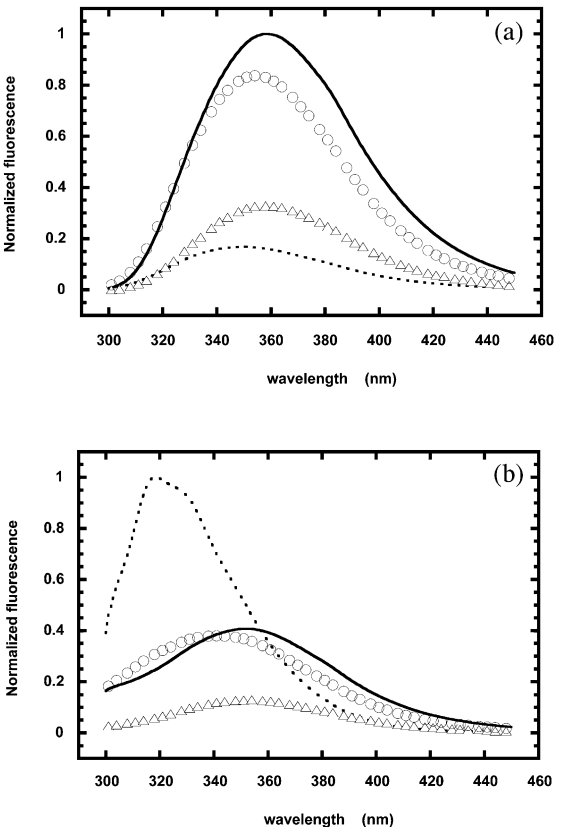

Fig. 2. Fluorescence spectra of fluorophores (a) 2.5 μM NATA and (b) 10 μg/ml TCAM. The emission spectra shown are of the particular flourophore in: 50 mM Tris buffer (*heavy solid line*), 4.0 M TMAO (*dashed line*), 400 mg/ml ficoll 70 (*open triangles*), and 400 mg/ml dextran 70 (*open circles*).

TCAM emission maximum (~12 nm), suggesting some degree of tryptophan burial within TCAM. The osmolyte, trimethylamine-*N*-oxide (TMAO) at a concentration of ≥2.7 M is known to restore TCAM to its fully folded state, and its emission spectrum is shown in Fig. 2b for reference [11]. Taken together, Fig. 2a,b provide suggestive evidence that 400 mg/ml dextran 70 may induce a detectable degree of structure in TCAM, but that ficoll 70 appears to have no effect at all on TCAM.

Using circular dichroism, evidence was sought of the appearance of secondary structure in TCAM induced by 400 mg/ml dextran 70. TCAM in the presence of 400 mg/ml dextran 70 shows an increase in negative ellipticity below 225 nm (see Fig. 3), a result often taken as evidence of a small increase in protein structural integrity. However, as also observed in Fig. 3, similar decreases in ellipticity were observed when (disulfide intact) RNase T1 was used in place of TCAM. It is highly unlikely that native RNase T1 assumes additional structure in the presence of dextran 70. If, as suggested by the fluorescence data, structure is induced into TCAM by dextran 70, CD appears not to be sensitive enough to detect these putative structural changes in the presence of 400 mg/ml crowding agent.

3.3. Quantification of folded TCAM using catalytic activity measurements

In the absence of TCAM or RNase T1, control experiments demonstrated no hydrolysis of the substrate, 20 mM 2′:3′ cGMP, as a function of time in buffered solutions containing either macromolecular crowding agents, buffer alone, 4 M TMAO or 8 M sarcosine. Enzyme specific activity values determined upon the addition of TCAM or RNase T1 to such solutions (final concentrations of 30 and 3 μg/ml, respectively) were obtained and then normalized to the specific activity of RNase T1 evaluated in buffered 20 mM 2′:3′ cGMP. These normalized specific activity values, expressed as percentage of the specific activity of RNase T1 in buffered 20 mM 2′:3′ cGMP, are given under column headings RNase T1 and

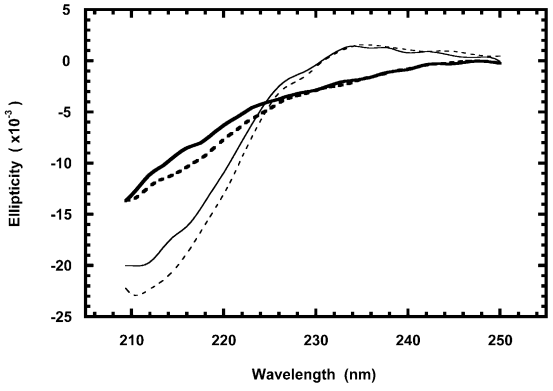

Fig. 3. Far-UV CD spectra of 0.34 mg/ml TCAM (*heavy lines*) or RNase T1 (*thin lines*) in 50 mM Tris buffer alone (*solid lines*) and in 400 mg/ml dextran 70 (*dashed lines*).

Table 1
Quantification of TCAM folding by specific activity measurements (50 mM Tris, 1 mM EDTA, pH 7.5, 22 °C)

Conditions	Relative specific activity (%)			Folded population
	RNase T1	TCAM	TCAM/ RNase T1	
Buffer alone	100	0	0	0
4 M TMAO	83.0	23.3	28.1	99.6
8 M sarcosine	67.1	18.9	28.2	100
300 mg/ml dextran 70	83.2	0.29	0.36	1.3
400 mg/ml dextran 70[a]	80.1	3.2	4.0	14.2
400 mg/ml dextran 70[b]	80.1	3.7	4.6	16.3
400 mg/ml ficoll 70	65.4	0.63	0.96	3.4
400 mg/ml BSA	107.4	0.40	0.37	1.3

Specific activity values were determined at each experimental condition reported and were normalized to the specific activity of RNase T1 obtained in buffer. The normalized specific activities × 100 are reported in the second and third columns. The ratios of data in these columns × 100, shown in column four, represent the percentage of active TCAM relative to active RNase T1 measured under the same experimental condition. The percentage folded population of TCAM was obtained by dividing the column listed as TCAM/RNase T1 by 28.2, then multiplying by 100.

[a] Results obtained using the limiting reaction velocity at time zero in Fig. 1.

[b] Results obtained using the limiting reaction velocity at time 1300 min in Fig. 1.

TCAM in Table 1. TCAM activity when added to buffered substrate solution was too low to be quantified. It is observed from Table 1 that the specific activity of RNase T1 in buffered 4 M TMAO containing substrate (pH 7.5) is 83% of that obtained in buffered substrate alone. The enzyme (RNase T1) is slightly more active in 400 mg/ml BSA (107%) than in the control, and is least active in 400 mg/ml ficoll 70.

In the presence of 4 M TMAO, TCAM is known to be fully folded and its specific activity under these conditions is 23.3% of the control (column 3 of Table 1). Comparison of the activity of fully folded TCAM in 4 M TMAO with that of RNase T1 in 4 M TMAO shows that TCAM is 28.1% as active as RNase T1 when both enzyme species are in their fully folded forms in 4 M TMAO (column 4 of Table 1). As is shown later, the osmolyte sarcosine forces TCAM to fold fully, and as in Table 1, fully folded TCAM again shows 28.2% of the activity of RNase T1 in 8 M sarcosine. The relationship of fully folded TCAM being 28% as active as RNase T1, regardless of the particular osmolyte present and despite the presence of very high osmolyte concentration, suggests this constant activity ratio could be used as a basis for evaluating the degree of folding of TCAM in the presence of macromolecular crowding agents. Assuming that the activity relationship that occurs in high concentrations of osmolytes holds also in the presence of macromolecular crowding agents, estimates of the percentage of the TCAM population that is catalytically active (i.e. folded) in the presence of crowding agent (column 5, Table 1) can be made from the results in Fig. 1 and the first four columns of Table 1.

3.4. Quantification of TCAM folding using crowding agents in osmolyte solutions

Naturally occurring osmolytes are known to force TCAM to fold into a functionally active 'native' ensemble. From the co-operative folding data, it is possible to determine ΔG^{o}_{N-D} using the linear extrapolation model. This Gibbs energy change is a measure of the stability of the protein in the limit of zero concentration of osmolyte. Fig. 4 shows a plot of the fluorescence ratio of TCAM as a function of the concentration of sarcosine, an osmolyte of the methylamine class. The solid line represents the non-linear least-squares best fit of the data using the linear extrapolation model, from which ΔG^{o}_{N-D} is evaluated. Thermodynamic reversibility of TCAM folding/unfolding in the presence of sarcosine is shown in Fig. 4 by demonstrating there is no hysteresis in the folding/

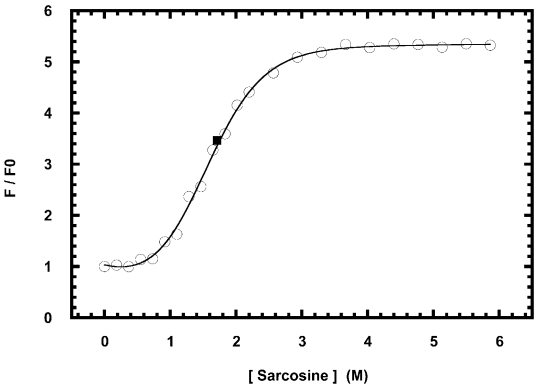

Fig. 4. Fluorescence ration of sarcosine-induced forced folding of TCAM (10 μg/ml) in the presence of 100 mM Tris buffer, pH 7.5 (278 nm excitation, 319 nm emission). The solid line represents the non-linear least-squares best fit of the data to the linear extrapolation model with $m = 1.33$ kcal/mol $\times$ M^{-1}, $\Delta G^{\circ}_{N-D} = -1.91$ kcal/mol and $C_{1/2} = 1.44$ M. The filled square is the result of a reversibility test as described in the text.

unfolding data. To illustrate this, a solution represented by a data point at 5.13 M sarcosine is diluted to a sarcosine concentration within the transition zone, and the fluorescence ratio (corrected for protein dilution) is determined. The filled square in Fig. 4 shows the result of such a measurement, demonstrating that at an osmolyte concentration in the transition zone the same fluorescence ratio is obtained, regardless of whether the experiment is performed in the folding or the unfolding direction. Close correspondence of the data obtained from both directions shows the lack of hysteresis and serves as a strong test of thermodynamic reversibility. Demonstration of thermodynamic reversibility eliminates the possibility of irreversible protein aggregation.

In order to evaluate the effect of the macromolecular crowding agent, 100 mg/ml dextran 70, on the stability of TCAM, sarcosine solutions were prepared such that they contained 100 mg/ml dextran 70 and 10 μg/ml (i.e. 0.9 μM) TCAM. These solutions (buffered in 1 mM EDTA, 100 mM Tris at pH 7.5) were allowed to incubate at 22 °C overnight; their fluorescence (excitation 278 nm, emission 319 nm) ratio was then determined and the data are shown in Fig. 5a. The same

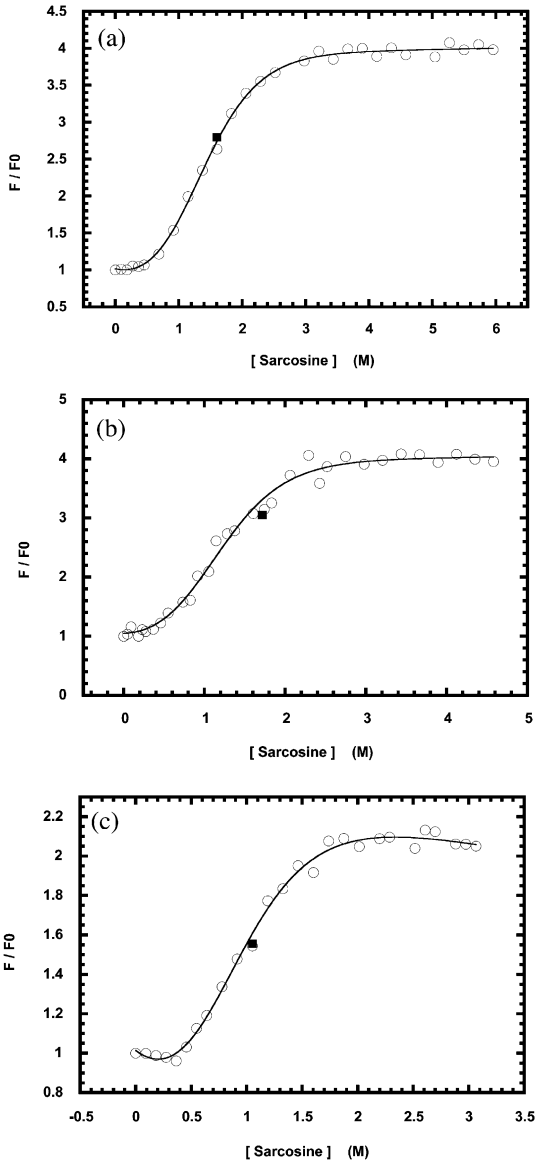

Fig. 5. Sarcosine forced folding of TCAM (10 μg/ml) in the presence dextran 70 at concentrations of (a) 100; (b) 200; and (c) 300 mg/ml. Except for the amounts of dextran 70, the experimental conditions are identical to those in Fig. 4. The filled square symbols are the results of reversibility tests as described in the text. Results of non-linear least-squares fits to the data (shown as solid lines) give respective *m*, apparent ΔG°_{N-D} and $C_{1/2}$ values of: (a) 1.25 kcal/mol $\times$ M^{-1}, -1.44 kcal/mol, and 1.15 M; (b) 1.37 kcal/mol $\times$ M^{-1}, -1.37 kcal/mol, and 1.0 M; (c) 1.58 kcal/mol $\times$ M^{-1}, -0.99 kcal/mol, and 0.63 M.

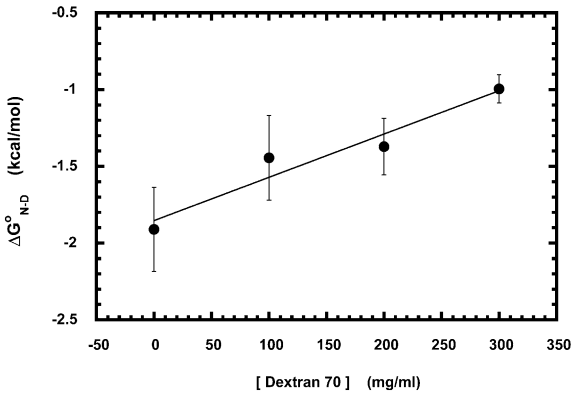

Fig. 6. *Apparent* ΔG^{o}_{N-D} values derived from the data in Figs. 4 and 5 as a function of dextran 70 concentration. The straight line is a linear fit of the data shown.

protocol was used to obtain folding data in the presence of 200 and 300 mg/ml dextran 70, giving the plots in Fig. 5b,c. Non-linear least-squares fits of the data to the linear extrapolation model gave *apparent* ΔG^{o}_{N-D} values that are presented as a function of dextran 70 concentration in Fig. 6.

4. Discussion

Very few experimental studies have attempted to evaluate the effects of macromolecular crowding on protein folding at room temperature, and fewer yet have tried to assess how much the agents shift the N↔D equilibrium [8–10,17]. The general conclusion arising from these studies is that the effect of macromolecular crowding on protein stabilization is small or non-existent. In order to evaluate the magnitude of the effect on the N↔D equilibrium, it is important to select a system that will enable detection of small increases in protein stabilization induced by macromolecular crowding. This means that the stability of the test protein must be marginal enough so that it is possible to determine measurable amounts of native and denatured protein. The easiest way of doing this is to select a system that is poised such that a significant region of the transition zone is experimentally accessible. The ΔG^{o}_{N-D} of the protein TCAM in neutral pH at 25 °C in aqueous buffer is of the order of −2 kcal/mol [11,12]. From theory, the proposed 10–100-fold increase in equilibrium constant for protein stability (amounting to 1.4–2.8 kcal/mol increase in protein stability) expected to arise from macromolecular crowding [3] should provide access to experimentally detectable amounts of native and denatured protein. Ideally, we would like to determine the shift in N↔D equilibrium that occurs in aqueous solution solely upon the addition of a macromolecular crowding agent. The N↔D equilibrium for reduced and carboxyamidated RNase T1 (TCAM) at pH 7.5 is 97% in the D form at 22 °C (see Fig. 4), making this system well suited for evaluating the effects of macromolecular crowding on the N↔D equilibrium. What is needed is a means of detecting a shift in the N↔D equilibrium or a way to quantify the fraction of N or D on addition of the crowding agent.

Spectral measurements provide the most common means of monitoring the shift in N↔D transitions. Taking advantage of the large differences in fluorescence between the folded and unfolded forms of TCAM, we have shown that naturally occurring osmolytes readily and reversibly shift the N↔D equilibrium to the native species [11]. Fluorescence-detected forced folding of TCAM by sarcosine (see Fig. 4) can be analyzed using the linear extrapolation model to give ΔG^{o}_{N-D}, a Gibbs energy change from which we determine that 97% of TCAM is in the D form under our experimental conditions. We have shown from analysis of TCAM forced-folding by sucrose, TMAO and sarcosine that the same ΔG^{o}_{N-D} is obtained [12]. The fact that ΔG^{o}_{N-D} is independent of osmolyte gives confidence in both the analysis and the use of osmolytes in evaluating protein stability. This demonstration of the ease of distinguishing between N and D forms using fluorescence, and the success in using osmolytes to evaluate protein stability, led to complementary approaches to assessing the effects of macromolecular crowding on protein stability. The approaches are: (1) to use fluorescence to quantify the shift in N↔D equilibrium induced by crowding agents alone; and (2) to use the linear extrapolation model for determining the effects of crowding agents on sarcosine-induced folding of TCAM.

Fig. 2a,b demonstrate the effects of macromolecular crowding agents on fluorescence spectral characteristics of TCAM. High concentrations of ficoll 70 show no change in the wavelength of maximum fluorescence for TCAM, while dextran 70 does show spectral shifts in the TCAM emission spectrum, consistent with the formation of some degree of folded TCAM species. Our tentative interpretation of these results is that ficoll 70 does not shift the D↔N equilibrium of TCAM and that dextran 70 only modestly shifts the equilibrium in favor of the native state. In order to have at least some estimate of the degree of stabilization offered by dextran 70, we turned to evaluating the effect of dextran 70 on sarcosine-induced stabilization of TCAM.

The presence of dextran 70 in sarcosine solutions shifts the midpoint of the transition to a lower $C_{1/2}$ value, a result consistent with dextran 70 being a protein stabilizer. By means of the linear extrapolation method, the apparent ΔG°_{N-D} is obtained as the Gibbs energy change for the N↔D equilibrium in the limit of zero sarcosine concentration [11,18]. Fig. 5a,b,c represent sarcosine-induced TCAM folding in the presence of fixed amounts (100, 200 and 300 mg/ml, respectively) of dextran 70. Thus, the apparent ΔG°_{N-D} values obtained from analyses of these data represent the stability of the N↔D equilibrium in the presence of the corresponding fixed concentration of dextran 70. The apparent ΔG°_{N-D} values as a function of dextran 70 concentration given in Fig. 6 clearly show that the negative ΔG°_{N-D} obtained in the absence of crowding agent becomes less negative as dextran 70 concentration is increased. This means that increasing concentrations of dextran 70 increase the stability of the TCAM native state. Extrapolation of the apparent ΔG°_{N-D} data to higher dextran 70 concentration gives an estimate of 22.4% for the native state population of TCAM in 400 mg/ml dextran 70. The increase in stability of TCAM in 400 mg/ml dextran 70 over the stability of TCAM in buffer alone [i.e. $\Delta\Delta G^{\circ}_{N-D} = \Delta G^{\circ}_{N-D}$ (400 mg/ml dextran 70) $- \Delta G^{\circ}_{N-D}$ (buffer)] indicates a 7.5-fold increase in the equilibrium constant for TCAM folding caused by the presence of 400 mg/ml dextran 70.

The assumption in evaluating protein stability increases due to dextran 70 as obtained from sarcosine folding experiments is that sarcosine and dextran effects on protein stability are additive. There is no easy way of testing this assumption. At this point, as for the conclusions drawn in other studies, the analysis indicates that macromolecular crowding effects are modest and at the low end of the estimates by Minton [3].

Without adequate spectral changes for use in directly determining the effects of dextran 70 on the TCAM folding equilibrium, we undertook an evaluation of the fraction of native protein in the presence of dextran 70. We took advantage of the observation that fully folded TCAM is 28% as active as RNase T1. Because this relationship holds under a variety of conditions in various osmolyte solutions (even at very high osmolyte concentration), we assume it will also hold in the presence of macromolecular crowding agents. In order to provide the most favorable conditions for producing folded TCAM, we used macromolecular crowding agents at the highest concentration (400 mg/ml) presumed to occur in cells. Catalytic activity in TCAM solutions (specific activity) is taken to be proportional to the folded population of TCAM. When this activity is compared with specific activity of RNase T1 under the same conditions and the activity relationship between TCAM and RNase T1 is considered, the percentage of the TCAM population in the native state can be estimated. The data in Table 1 show native state (folded) populations of 1.3, 3.4 and 16.3% for TCAM in the presence of 400 mg/ml BSA, ficoll 70 and dextran 70, respectively. The lack of correspondence in (folded) TCAM native state populations among the various crowding agents suggests that macromolecular crowding agents should not be considered as merely inert crowding objects. As others have stated, it is highly likely that the crowding agents affect the protein system in ways other than simple excluded-volume effects [4,8,17].

The catalytic activity experiments and the $\Delta\Delta G^{\circ}_{N-D}$ experiments present a basis for comparison of protein stabilization of TCAM in the presence of 400 mg/ml dextran 70. The fact that both methods show similar percentages of native

TCAM in the presence of 400 mg/ml dextran 70 (16% native in dextran 70 alone and 22% native in the ΔG^{o}_{N-D} experiments) provides some confidence in the conclusion that macromolecular crowding has only a modest influence on the TCAM folding equilibrium. Knowing the ΔG^{o}_{N-D} of TCAM in buffer alone (see Fig. 4 legend) and the fraction of folded TCAM in 400 mg/ml dextran 70 from catalytic measurements (16%), we can calculate a five-fold increase in folding equilibrium constant due to macromolecular crowding. Both methods point to an approximate 5–7.5-fold increase in the folding equilibrium constant of TCAM in the presence of 400 mg/ml dextran 70.

Given the numerous examples showing that macromolecular crowding strongly promotes protein–protein interactions and protein aggregation [2,5,7,9,17,19], avoiding aggregation becomes a project in its own right. Two TCAM concentrations were used in these experiments (30 and 10 μg/ml). The light scattering and catalytic activity measurements were performed at the higher concentration, while the lower concentration was used in the sarcosine forced-folding experiments. Light scattering by aggregates is proportional to the reciprocal of the fourth power of the wavelength, but despite using short wavelengths, our light scattering measurements showed no evidence of particles large enough to scatter light. Negative evidence however, does not permit exclusion of the possibility of aggregation.

In contrast to light scattering measurements, the product vs. time plot for 2′:3′ cGMP-catalyzed hydrolysis in 400 mg/ml dextran 70 provides evidence of what appears to be a slow increase in the amount of folded TCAM over a 24-h period. Based on limiting slopes of the data in Fig. 1 at time zero and after 20 h (1300 min), the apparent increase in active enzyme after 20 h only amounts to approximately 15% more than is present at time zero. The additional active enzyme could possibly arise from the dissociation of putative TCAM aggregates, or perhaps from recovery of TCAM associated with the dextran 70. Whatever the source, the result demonstrates (in agreement with others) the difficulty in obtaining unambiguous results with crowding agents [4,8,17].

The fact that protein folding/unfolding is reversible in the presence of 0, 100, 200 and 300 mg/ml dextran 70 and sarcosine provides strong evidence that significant TCAM irreversible aggregation has not occurred in these solutions. In obtaining folding data near the midpoint of a folding transition from the folding direction, the TCAM protein used is initially in the denatured state and, on shifting to the $C_{1/2}$ concentration, the protein relaxes to a N/D equilibrium near the 50:50 mixture of native and denatured protein. By contrast, in obtaining data at the transition midpoint from the unfolding direction, the TCAM at high osmolyte concentration exists in its folded state initially and, on shifting to the $C_{1/2}$ concentration, denatures to an equilibrium position near a 50:50 N/D mixture. Due to the high initial concentration of denatured TCAM, irreversible aggregation of TCAM would be much more prone to occur in the folding than in the unfolding direction, making it unlikely to obtain the reversible data shown in Figs. 4 and 5. Moreover, it is clear that the percentage of native protein determined by means of functional activity in 400 mg/ml dextran 70 is close to that determined from the Gibbs energy measurements presented. This close correspondence in the fraction of native and denatured species from two very different methods of determination suggests that significant aggregation is an unlikely process of importance in the presence of dextran 70.

The experiments provided here attempt to obtain some thermodynamic measure of the stabilization of proteins due to macromolecular crowding, and the results suggest the effects are small. The fact is that crowding of TCAM by BSA (68 kDa) and ficoll 70 appears to give much smaller stabilization effects than dextran 70 does. We attribute the discrepancies in effectiveness of the various crowding agents as due to crowding agent-specific effects. If this is the case, it will not be easy to segregate crowding effects from various possible specific effects the individual crowding agents may impose on the protein system under study. Are there other 70-kDa crowding agents that would shift the D↔N equilibrium more than dextran 70? Given the heterogeneity of macromolecular crowding agents in a cell, will factors other than exclud-

ed volume generally tend to diminish the one–two order-of-magnitude effects on the D↔N equilibrium expected on the basis of hard-sphere crowding theory? Answering these questions will require much more vigorous and extensive experimentation than has occurred to date.

Acknowledgments

Supported by US Public Health Research Grant GM49760.

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