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A Compact Monomeric Intermediate Identified by NMR in the Denaturation of Dimeric Triose Phosphate Isomerase

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The denaturation of triose phosphate isomerase (TIM) from *Saccharomyces cerevisiae* by guanidine hydrochlorids at pH 7.2 has been monitored by NMR spectroscopy in conjunction with optical spectroscopy. In the absence of denaturant, the hydrodynamic radius of 29.6(±0.25) Å and the substantial chemical shift dispersion evident in the NMR spectrum are consistent with the highly structured dimeric native state of the protein. On the addition of 2.2 M guanidine hydrochloride the effective hydrodynamic radius increases to 51.4(±0.43) Å, consistent with that anticipated for the polypeptide chain in a highly unstructured random coil state. In 1.1 M guanidine hydrochloride, however, the effective hydrodynamic radius is 24.0(±0.25) Å, a value substantially decreased relative to that of the native dimeric state but very close to that anticipated for a monomeric species with native-like compaction (23.5 Å). The lack of chemical shift dispersion indicates, however, that few tertiary interactions persist within this species. Far UV CD and intrinsic fluorescence measurements show that this compact intermediate retains significant secondary structure and that on average the fluorophores are partially excluded from solvent. Such a species could be important in the formation of dimeric TIM from its unfolded state.

Keywords: protein denaturation; TIM barrel; NMR; hydrodynamic radius; protein dimerisation

Triose phosphate isomerase (TIM) has a three-dimensional structure consisting of eight parallel, hydrogen bonded β-strands at the core of the molecule; each strand is connected by a loop and one or two helices which pack against the barrel surface. The TIM barrel fold appears to serve as a scaffold for a diverse range of enzymatic functions and has a large representation in the structural database (22 superfamilies adopting the TIM barrel fold in the SCOP database v1.48 (Murzin *et al.*, 1995)). The relative abundance within the database has led to a proposal that the architecture is favoured perhaps in terms of its stability or folding properties (Orengo *et al.*, 1994).

Size exclusion chromatography and ultracentrifugation experiments indicate that the native state of TIM in solution is a dimer (McVittie *et al.*, 1972; Zabori *et al.*, 1980). Moreover, investigations of the concentration dependence of TIM folding have established a clear link between dimer formation and isomerase function (Waley, 1973). In accord with these observations, the three-dimensional structure from crystallographic studies reveals a dimeric fold, and shows that the active site pocket is close to the dimer interface (Lolis *et al.*, 1990). The latter comprises four of the eight loops that immediately follow the β-strands of the barrel. The

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Abbreviations used: AAT, aspartate aminotransferase; ANS, 8-anilino-1-napthalenesulphonic acid; αTS, α-subunit of tryptophan synthase; CD, circular dichroism; DTT, dithiothreitol; FID, free induction decay; GuDCl, guanidine deuterochloride; GuHCl, guanidine hydrochloride; ppm, parts per million; TIM, triose phosphate isomerase.

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importance of specific interactions at the interface has been elegantly demonstrated by mutations to the wild-type sequence of trypanosomal TIM. For example, a seven residue deletion (Borchert *et al.*, 1994) or the mutation of two residues in the main interface loop results in a stable monomeric form of TIM (Schliebs *et al.*, 1997). The major effect of disrupting a relatively small number of interactions by such mutations, and the high level of complementarity at the interface (Jones & Thornton, 1995), suggest that a considerable degree of subunit folding is required for dimerisation and raises questions about the nature of structures formed during folding.

Here, we describe biophysical studies of the unfolding of TIM from *Saccharomyces cerevisiae*. The major aim of this work has been to monitor the loss of secondary and tertiary structure during the denaturation of TIM in conjunction with measurements of the molecular dimensions. ^{1}H NMR spectra (Figure 1) readily allow the denaturation of TIM by guanidine deuterochloride (GuDCl) to be monitored. In the absence of denaturant (upper trace, Figure 1), there is a significant degree of chemical shift dispersion. Although assignments of specific resonances in the spectrum are not known, it is likely that the perturbation of resonances to positions outside the envelope of random coil chemical shift values can be attributed, in most part, to localised ring current effects from aromatic residues (Wüthrich, 1986). For example, peaks close to 0 ppm are likely to correspond to protons of side-chain methyl groups in close proximity to aromatic residues. Substantial chemical shift dispersion of this type is characteristic of a highly ordered native structure. Addition of even moderate concentrations of denaturant, however, results in the loss of this dispersion. In the presence of 2.2 M GuDCl (lower trace, Figure 1), the spectrum resembles closely the chemical shift pattern of a highly unfolded polypeptide chain (Wüthrich, 1986).

To probe the changes in molecular dimensions during the denaturation of TIM, NMR diffusion measurements were conducted (Figure 2) using pulse-field gradient methodologies (Gibbs & Johnson, 1991; Altieri *et al.*, 1995; Dingley *et al.*, 1995). These approaches have been employed recently in studies of protein self-association (Lin & Larive, 1995) and protein folding and unfolding (Jones *et al.*, 1997; Pan *et al.*, 1997; Wilkins *et al.*, 1999). Since the viscosity of aqueous solutions increases upon addition of GuDCl, a small molecule, dioxan, was used as an internal standard (Jones *et al.*, 1997; Wilkins *et al.*, 1999). Values of the effective hydrodynamic radius of the protein were extracted by comparing the rates of decay of the protein and dioxan resonances with increasing gradient strength. Relationships have been established previously between the effective hydrodynamic radius (R_h) and the number of amino acid residues in a polypeptide chain (N) for proteins adopting compact globular native folds

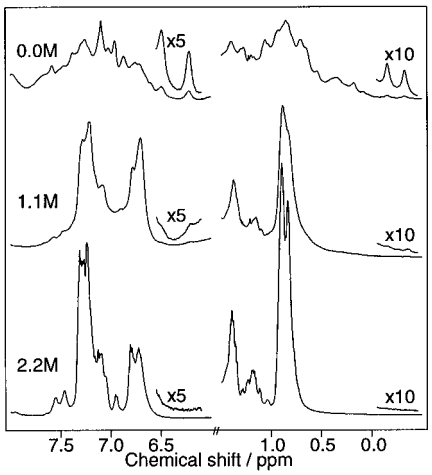

Figure 1. ^{1}H NMR spectra of 0.6 mM TIM from *S. cerevisiae* (Sigma Chemical Company), 5 mM d_{11} Tris, 100 mM NaCl (pH 7.0), 99.9 % ^2H_2O (concentration of denaturant, GuDCl, as indicated). A crystalline suspension of TIM containing 20 mg/ml was buffer exchanged, concentrated and divided equally in two parts. NMR experiments were conducted at 25 °C with two solutions of the same protein content, containing either 0.0 or 2.2 M GuDCl. Each sample contained additionally 0.03 % dioxan as a chemical shift reference and internal standard for diffusion measurements. Samples of TIM containing GuDCl concentrations between 0.0 and 2.2 M GuDCl were prepared by swapping equal volumes between the two samples. A small amount of precipitate was observed at 0.5 M GuDCl which was readily soluble at higher concentrations of denaturant. For this reason the spectra were normalised before the intensities of resonances in the NMR spectra of different samples were compared. In particular the total protein concentration was assumed to be linearly proportional to the intensity of the aliphatic region and spectra were normalised accordingly. A home-built NMR spectrometer operating at 500.1 MHz was used to record FIDs comprising 256 transients of 8192 complex points and a dwell time of 125 μs. 1D spectra were generated from the FID using standard processing methods. After the completion of 1D NMR, the hydrodynamic radius was measured by pulse-field gradient NMR methods (see legend to Figure 2 for details).

($R_h = (4.75 \pm 1.11)N^{0.29 \pm 0.02}$) and under strongly denaturing conditions ($R_h = (2.21 \pm 1.07)N^{0.57 \pm 0.02}$; Wilkins *et al.*, 1999). Values predicted from these relationships have been used to aid the interpretation of the TIM experimental data. Thus the measured hydrodynamic radius of 29.6(±0.25) Å for TIM in the absence of denaturant (Figure 3(a)) is consistent with an estimate of 28.7 Å for a compact polypeptide chain of 494 amino acid residues (Wilkins *et al.*, 1999), confirming that under these conditions TIM is a dimer. The addition of concentrations of GuDCl up to ca. 1.1 M is accompanied by a substantial decrease in the effective hydrodynamic radius (Figure 3(a)). The value measured in 1.1 M GuDCl is 24.0(±0.25) Å, which is very close

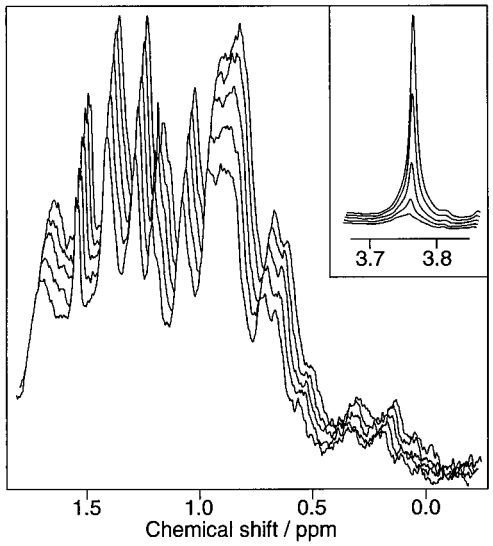

Figure 2. ^{1}H NMR spectra of 0.6 mM TIM in 0.0 M GuDCl (solution conditions as in Figure 1) acquired with a PG-SLED sequence (Jones *et al.*, 1997) with additional water suppression achieved by saturation of the HO^{2}H resonance. The diffusion labelling gradient was varied linearly from 5% to 100% full gradient strength, in 5% increments. Each FID was recorded as a sum of 32 transients and for each sample, at least three sets of FIDs, encompassing the full range of gradient strengths, were recorded. For clarity, only traces at 20, 40, 60, 80 and 100% full gradient strength are shown (stacked front to back). The intensity of the NMR signal depends on the gradient strength and the diffusion constant, *D*, which is inversely proportional to the viscosity of the solution. Since the viscosity of aqueous solutions increases upon addition of GuDCl, a small molecule, dioxan, was used as an internal standard. The rates of decay of the intensities of the protein and the dioxan resonances were obtained by fitting to Gaussian functions (a double Gaussian was used for signals arising from the overlap of dioxan and protein resonances). From the ratio of the protein and dioxan decay rates an effective hydrodynamic radius for the protein was extracted assuming an effective hydrodynamic radius of 2.12 Å for dioxan (Wilkins *et al.*, 1999). Inset: Resonance corresponding to the internal standard, dioxan. Note that the decay of the dioxan signal is much faster than the decay of TIM signals as dioxan molecules diffuse more rapidly than the protein molecules.

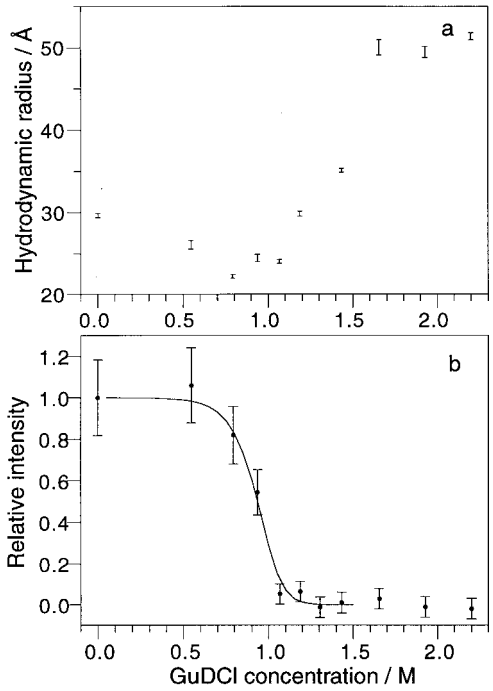

Figure 3. (a) Variation in the effective hydrodynamic radius of TIM with GuDCl (solution conditions as for Figure 1). For each GuDCl concentration the value given is an average of those extracted from three experiments recorded using the same protein sample over a period of 36 hours. (b) Mean normalised intensity of two resolved ^{1}H resonances (at −0.2 and 6.2 ppm) *versus* GuDCl concentration. The intensities were determined using FELIX software, version 2.3 (Hare Research). The continuous line corresponds to a representative outcome of fitting the NMR intensity to a model in which the free energy of TIM dimers and monomers vary linearly as a function of GuDCl concentration (Greene & Pace, 1974). The relatively few data points defining the transition region preclude a more complete thermodynamic analysis. The dissociation constant obtained from the NMR data is, however, in the range 1×10^{-12} to 5×10^{-14} M. Interestingly this is similar to the dissociation constant estimated for dimeric yeast TIM from the crystal structure on the basis of buried surface area (10^{-14} to 10^{-16} M; Lolis *et al.*, 1990).

to that expected for a compact monomeric protein of 247 residues in length (23.5 Å). Further addition of GuDCl, to 2.2 M, results in a large increase in the effective hydrodynamic radius to 51.4(±0.43) Å. This is consistent with the value of 51.1 Å expected for a denatured TIM monomer of 247 residues (Wilkins *et al.*, 1999).

Assuming that the dispersion of resonances in the NMR spectrum arises from the tertiary interactions of the native dimer, it follows that the intensities of resonances which lie outside the envelope of random coil chemical shifts provide a direct measure of the concentration of native-like TIM molecules. Figure 3(b) shows the variation in the mean intensity of two such resonances as a function of GuDCl concentration. The decrease in native-like signal intensity is complete by ca. 1 M GuDCl and mirrors closely changes in the molecular size (Figure 3(a)). The spectrum obtained in 1.1 M GuDCl (Figure 1, middle trace) is, however, distinct from that expected for a highly disordered and unfolded protein. The broad lineshapes observed in the ^{1}H spectrum at concentrations of GuDCl between ca. 0.9 and 1.4 M suggest that in the monomeric intermediate there are slow fluctuations between conformationally distinct states, such as have been observed in a range of partially

folded molten globule states (Baum *et al.*, 1989; Shortle, 1996). At higher denaturant concentrations, a substantial reduction in the linewidths of the NMR resonances is observed (Figure 1), the narrow lines suggesting that the various polypeptide conformations interconvert rapidly, as expected for a highly unfolded species. In summary, therefore, the NMR measurements suggest that the denaturant acts to dissociate TIM dimers to compact monomers which then become increasingly disordered in the presence of increasing concentrations of denaturant.

In order to characterise further the partially structured TIM monomer, intrinsic fluorescence and far UV CD spectra were acquired at 1.1 M GuHCl. As the NMR data suggest that under these conditions TIM is effectively completely in monomeric form, the optical data can be compared with the NMR data even though the protein concentrations used in the two sets of experiments are substantially different. The far UV CD spectrum (Figure 4(a)) indicate that a significant proportion of the native-like secondary structure is present, and fluorescence measurements (Figure 4(b)) show that at 1.1 M GuHCl there is a blue-shift of the emission spectrum relative to the spectra acquired at higher denaturant concentrations. We conclude that the monomeric intermediate detected by NMR contains considerable secondary structure and that on average the tryptophan residues are in local environments that are at least partially hydrophobic in character (Eftink, 1991). Under solution conditions favouring monomeric TIM, an enhancement and blue shift of the fluorescence spectrum of the hydrophobic dye ANS (8-anilino-1-napthalenesulphonic acid) is observed (Figure 4(c)). This is indicative of solvent-accessible hydrophobic regions and is not observed under solution conditions favouring TIM dimers or fully unfolded TIM monomers.

In conclusion we have identified a partially folded monomeric intermediate in the denaturation of dimeric TIM. It is highly compact and possesses significant secondary structure yet lacks persistent tertiary interactions. It is interesting to compare these results with those from studies of other eight-stranded β-barrels. In the case of the monomeric protein indole-3-glycerol-phosphate syn-thase, a partially folded intermediate has been identified (Sanchez del Pino & Fersht, 1997). For tetrameric ($\alpha_2\beta_2$) tryptophan synthase from *Escherichia coli*, the α-subunit (αTS) is monomeric and retains its structure and enzyme activity in the absence of the β-subunit (Miles *et al.*, 1987). Denaturation of αTS by urea proceeds *via* a partially folded intermediate, present in 3.2 M urea, in which the helical secondary structure is lost although the core β sheet and much of the tertiary packing is retained (Gualfetti *et al.*, 1999a). Hydrodynamic radius measurements show, however, that in contrast to the results reported here for TIM, the partially folded intermediate formed under these conditions is significantly expanded

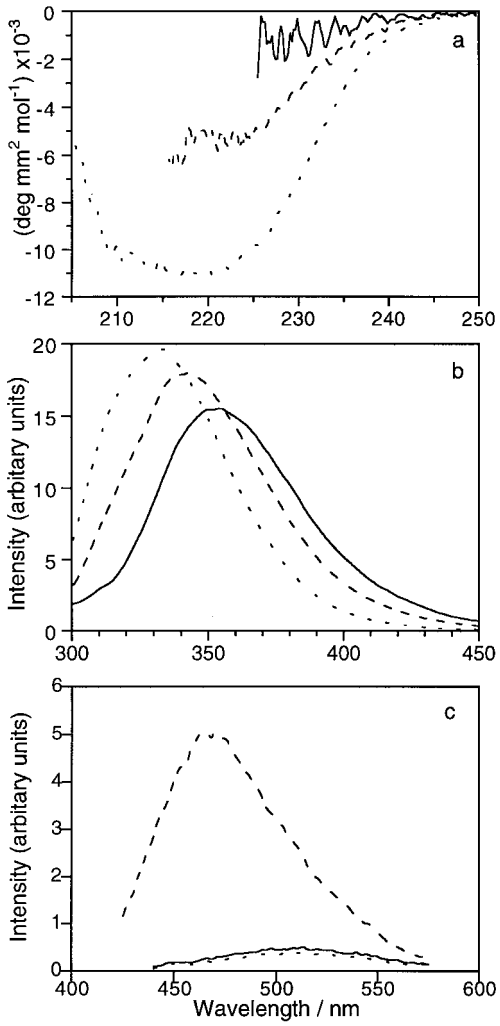

Figure 4. (a) CD spectra of 8 μM TIM, 25 °C, pH 7.2 (- - 0.0 M, — 1.1 M and −2.6 M GuHCl). Spectra were recorded as the average of two scans using a Jasco 720 spectropolarimeter and a 1 mm pathlength quartz cuvette. (b) Fluorescence emission spectra of 8 μM TIM, 25 °C, pH 7.2 (- - 0.0 M, — 1.1 M and −2.6 M GuHCl). Spectra were recorded as the average of eight transients using a PE LS50B fluorimeter. Emission and excitation slit widths were 2.5 nm and an excitation wavelength of 280 nm was selected. (c) Fluorescence emission spectra of 86 μM ANS, 10 μM TIM, 25 °C, pH 7.2 (- - 0.0 M, — 1.1 M and −2.6 M GuHCl). An excitation wavelength of 390 nm was selected. In each case the protein solutions contained 100 mM Tris, 100 mM NaCl, 5 mM EDTA and 1 mM DTT and were incubated at 25 °C, for 24 hours, in a circulating water bath. During measurements, the cuvette temperature was regulated by a circulating water bath and a thermocouple was used to determine the temperature of the solution within the cuvette.

with an effective hydrodynamic radius of ∼38 Å in contrast to the value for the native state of ∼24 Å (Gualfetti *et al.*, 1999b).

The results reported here for dimeric TIM from *S. cerevisiae* are, however, similar to those from a study of the denaturation of *E. coli* aspartate ami-

notransferase (AAT). This dimeric α/β protein adopts the PLP-dependent transferase fold which consists of a seven-stranded β-barrel rather than the eight-stranded TIM fold. For AAT, unfolding occurs *via* a monomer which is shown to be compact by ultracentrifugation and gel filtration studies and to retain a degree of native-like secondary and tertiary structure (Herold & Kirschner, 1990). The presence of compact monomeric intermediates in the denaturation of these dimeric proteins is consistent with a proposed general mechanism for the formation of oligomers from unfolded polypeptides. In this model, the formation of assembly-competent conformations precedes the association of subunits (Jaenicke, 1987). The exact nature of the assembly-competent subunit will depend on the type of fold and the extent of interdigitation between subunits. In the case of TIM, we suggest that the formation of compact monomers could be pre-requisite for dimerisation.

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