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Tumour necrosis factor is in equilibrium with a trimeric molten globule at low pH

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Abstract

The reversible acid denaturation of tumour necrosis factor, TNFα, a trimeric, all-β protein, leads to significant conformational changes within the molecule. A change in far UV CD spectra reveals a shift in the secondary structure content of the protein, with α-helical structure being induced. Loss of ellipticity in the near UV reflects a loss of tertiary interactions. This form of TNF is both compact and trimeric, as revealed by fluorescence anisotropy and sedimentation velocity analysis, respectively. Acid-denatured TNF therefore possesses the defining features of the molten globule intermediate while retaining the ability of the still incompletely folded monomers to exhibit the surface specificity necessary for maintaining the trimeric state.

Key words: Circular dichroism; Fluorescence; Protein folding; Tumor necrosis factor; Membrane insertion

1. Introduction

Studies of the in vitro re-folding of monomeric, globular proteins have demonstrated the involvement of the condensed molten globule as both a kinetic and an equilibrium intermediate [1–3]. It has also been proposed that its properties would fit it for involvement in translocation across biological membranes [4]. Indeed, the acid-induced molten globule form of colicin A, a pore-forming toxin, is proposed to be the form involved in membrane insertion [5]. Tumour necrosis factor-α (TNFα) is a globular, trimeric protein [6] consisting of identical 17 kDa subunits. It is an all-β protein, with a 50% β-sheet content, as determined from its crystal structure as well as by circular dichroism (CD) [6–8]. Maintenance of the trimeric structure is essential for its activity [9–11]. On reducing the pH of the solution, TNF inserts into lipid bilayers, thereby acting as an ionophore [12]. We present evidence that the acid-denatured form of TNF, termed TNF-A, is a trimeric molten globule. This form of the trimer appears to be involved both in membrane insertion and, in the similar, neutral pH form, as an intermediate in the folding and assembly pathway of TNF.

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Abbreviations: CD, circular dichroism; GdmCl, guanidinium chloride; TNF, tumour necrosis factor.

2. Materials and methods

2.1. Circular dichroism

CD spectra were acquired using a Jobin-Yvon Dichrographe VI. Native TNF was in 50 mM sodium phosphate, pH 8.0, and the acid-denatured protein, was obtained by a 10-fold dilution of native TNF into 0.1 M KCl/HCl, pH 1.0, giving a final pH of 1.6. Protein concentrations were in the range 0.31–0.51 mg · ml^{-1} (17.9–29.4 μM monomer). For far and near UV CD spectra, the respective cell pathlengths were 0.1 mm and 1 cm. Spectra were recorded at 20°C. Secondary structure content was evaluated according to Provencher and Glöckner [13].

2.2. Steady-state fluorescence measurements

Fluorescence data were collected using a Perkin Elmer MPF3-L spectrofluorimeter. Emission spectra were collected using an excitation wavelength of 280 nm, with bandwidths in the range 16–24 nm. Spectra were collected at 20°C. The protein concentration was 30 μg · ml^{-1} (1.73 μM monomer). Solution conditions for both the native and acid-denatured forms of the protein were as for the CD measurements.

The kinetics of re-folding of GdmCl unfolded and acid-denatured TNF were determined using excitation and emission wavelengths of 280 and 320 nm, respectively. TNF was acid denatured for 10 min at room temperature by diluting the native protein 10-fold into 0.1 M KCl/HCl, pH 1.0, giving a final pH of 1.6, or unfolded in GdmCl for 10 min at room temperature. In both cases, the protein was re-folded at a concentration of 30 μg · ml^{-1} (1.73 μM monomer), by diluting into 50 mM phosphate buffer containing GdmCl to give a final concentration of 0.5 M GdmCl at pH 7.6. The temperature of re-folding was 5°C. The values of the rate constants were determined as the mean of three measurements ± the standard deviation.

2.3. Fluorescence lifetime measurements

The decay of tryptophan fluorescence was measured using an Edinburgh Instruments 199 T-geometry lifetime spectrofluorimeter with single photon counting. The nanosecond flashlamp was operated at 50–60 kHz in 0.5 bar hydrogen, giving pulse widths of less than 1.5 ns duration. The excitation wavelength was 330 nm, with the slit bandwidth at 30 nm. The decay data were analysed using an iterative non-linear least squares reconvolution procedure in which the lamp profile, recorded before protein measurements were taken, as well as the whole decay, were fitted to one, two or three exponentials.

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2.4. Sedimentation analysis of acid-denatured TNF

Differential sedimentation velocity analysis was performed using a Beckman Model E analytical ultracentrifuge with Schlieren optics. TNF was acid denatured by overnight dialysis against a KCl/HCl buffer, pH 1.0, at room temperature. Sedimentation velocity runs of the protein were performed at two different protein concentrations, with one sample in a wedge window cell, and the other in a standard cell, with both in the same rotor. The rotor speed was 60,000 rpm at 22.9°C. The sedimentation constant, s°20,w, was derived using the procedures of Shumaker and Adams [14].

3. Results

3.1. CD spectra

On lowering the pH there is a significant change in the secondary structure of the protein (Fig. 1a). Analysis of the spectra shows that the acid and native (in parentheses) states exhibit 18% α-helix (2%), 49% β-sheet (55%), and 33% remainder (43%). Most of the increase in helix is at the expense of aperiodic structure in the native state. Since CD correctly predicts the secondary structure content of the native protein [6] and since there is no conformational contribution of aromatic residues to the near UV CD in the acid state (Fig. 1b), there is no possibility in this case of artefacts arising from aromatic contributions to the far UV CD spectra.

The near UV CD spectrum of TNF at low pH reveals a lack of asymmetry in the environment of the aromatic amino acid residues (Fig. 1b), indicating the loss of persistent tertiary structure. TNF-A thus possesses a different conformation from that of the native state.

3.2. The compactness and quaternary structure of TNF-A

In addition to increased rotational freedom in TNF-A, the tryptophan residues are seen to be more accessible to solvent than in the native state but still partially shielded compared with the unfolded state. The wavelength of maximum fluorescence emission shifts from 320 nm to 336.5 nm, accompanied by a reduction in the intensity (Fig. 2).

The fluorescence anisotropy decays of native, acid-denatured and GdmCl-unfolded TNF were measured (Fig. 3). In each case, the data were best fitted to a single component decay. For native TNF, the experimentally observed rotational correlation time is close to the predicted value, despite the inherent assumptions in the calculation (Table 1). The rapid decay observed for the

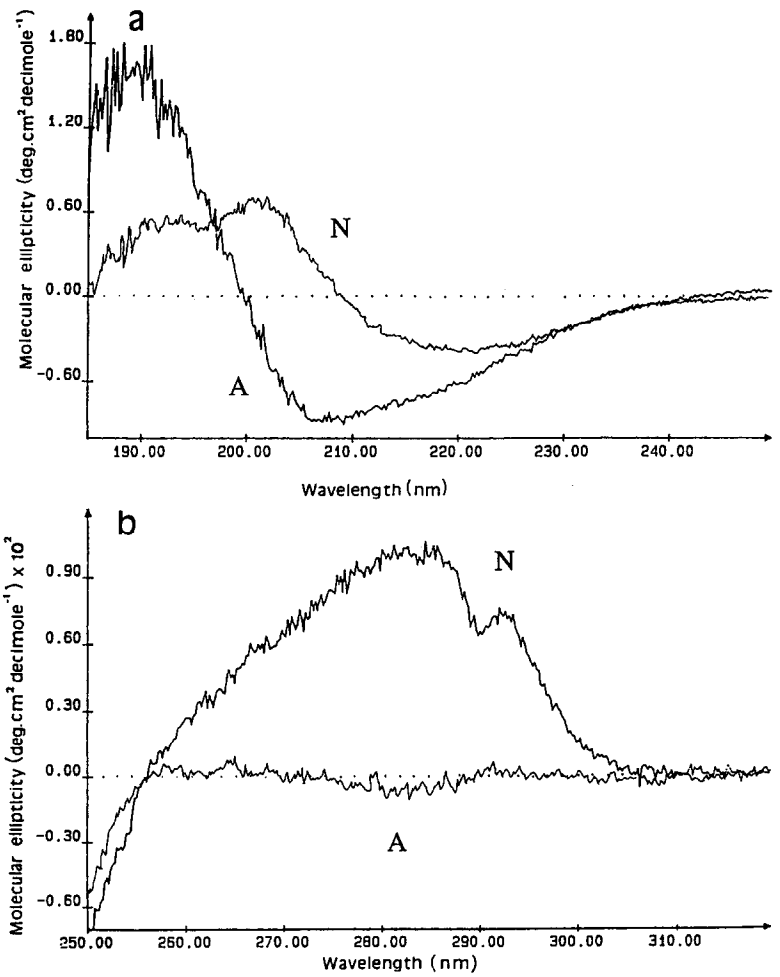

Fig. 1. CD spectra of native (N) and acid-denatured (A) TNF. (a) Far UV and (b) near UV CD of native and acid-denatured TNF.

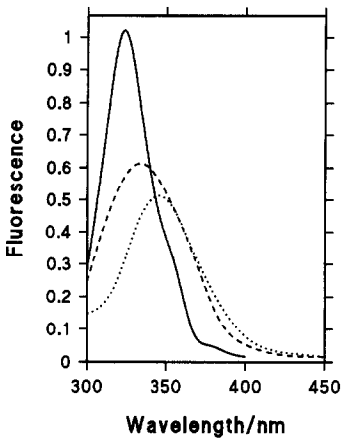

Fig. 2. Fluorescence spectra of native (—), GdmCl-unfolded (····) and acid-denatured (– – –) TNF.

unfolded protein is typical of a freely rotating tryptophan [15]. The decay for TNF-A is probably complex but the precision of the data only allows one component to be fitted. The value of the (average) rotational correlation time is intermediate between those for the native and unfolded proteins. The tryptophan residues are thus more mobile in TNF-A than in native TNF, presumably associated with increased dynamics and a slight expansion of the protein structure.

The alternative explanation of the above results is that the protein dissociates under acid conditions into folded monomers. This was investigated by sedimentation velocity experiments, leading to a value of the sedimentation constant, s°20,w, of 3.98 S. From a plot of log M_r against s°20,w for a number of well-characterised, globular (i.e. compact) proteins, a molecular weight of 56 kDa is obtained, demonstrating that TNF-A is both trimeric and compact. Together with the spectroscopic evidence, therefore, TNF-A is shown to exhibit the characteristics of a molten globule [2,3].

3.3. Re-folding of TNF-A and the GdmCl-unfolded protein

The kinetics of the rate-determining steps of re-folding from both TNF-A and the GdmCl-unfolded protein have been determined and shown to be the same under identical folding conditions (Fig. 4). The recovery of native fluorescence is described in both cases by a biphasic process. The first phase is complete within the dead-time of a manual mixing experiment. The first order rate constants obtained for the slow phase (Fig. 3b) are 0.25 ± 0.03 min^{-1} and 0.23 ± 0.03 min^{-1} for re-folding from the acid- and GdmCl-denatured states, respectively.

The rate-limiting step of folding from the GdmCl-unfolded state is first order and slower than the rate of association, as measured by chemical cross-linking (Hlodan and Pain, in preparation). The identity of the kinetics for the rate-determining step is strong evidence, therefore, that re-folding udner physiological conditions takes place, like that from TNF-A, through an already associated, condensed but 'molten' intermediate (I_3):

$$3M \rightarrow \rightarrow I_3 \rightarrow N_3$$

4. Discussion

The notable finding is that a multi-subunit protein can be partially denatured to a molten globule whilst retaining its oligomeric structure. In general, molten globule states have been well characterised only for monomeric proteins [1,3,16]. However, upon renaturation from low temperatures, glyceraldehyde-3-phosphate dehydrogenase from a hyperthermophilic organism exhibits a stable state that is tetrameric, has native-like secondary structure and a non-native environment of aromatic residues [17]. The dimeric enzyme, creatinase, in which quaternary structure was preserved, has been found to undergo irreversible denaturation to an acid molten globule intermediate [18].

Unlike the folding of monomeric proteins, studies of the assembly of multi-subunit proteins [19] have not so far pin-pointed at which, if any, stages of folding and assembly the molten globule is involved. A key question that remains to be answered concerns the degree of specificity required for the initial association of the folding monomers. Despite the lack of persistant tertiary structure, inter-subunit contacts between the polypeptide chains of TNF are retained and the protein can re-fold to the native state from this intermediate.

The results presented here add further weight to the growing evidence for a wide-ranging role for the molten globule in vivo [20]. An acid-denatured state of TNF has recently been shown to insert into lipid bilayers, with the

Table 1
Rotational correlation times of native and denatured TNF

	Rotational correlation time (ns)
Predicted value for protein of 52 kDa (assuming a spherical model)	21.5
native TNF	18.7 ± 3.3
TNF-A	11.8 ± 0.7
GdmCl-unfolded TNF	2.95 ± 0.75

The predicted rotational correlation time (τ) for native TNF, assuming it to be a hydrated sphere, is described by the following equation:

$$\tau = \frac{h \cdot M_r \cdot (\bar{v} + h)}{RT}$$

where h = solvent viscosity, M_r = molecular weight of the protein, $\bar{v}$ = its partial specific volume, and h = degree of hydration, typically 0.2 g H_2O per g protein.

TNF retaining its trimeric structure within the membrane [12]. Taken with these results, the intermediate on the pathway of insertion is seen to be a trimeric molten globule. The induction of helix shown here in TNF-A is potentially significant, as α-helices have been proposed to be important for the insertion of proteins into membranes [20], although the specificity leading to association of the monomers to a trimeric molten globule is retained.

One question which arises concerns the relevance of acid-denatured states of proteins generated in vitro to the situation that may occur in the cell. It is clear that there are differences between the pH of the bulk solution and that of the local environment that facilitates the insertion of proteins into membranes. For example, the in vitro reconstitution of the translocation apparatus in *E. coli*, of which integral membrane proteins are a constituent, was favoured using bilayers having an acidic lipid composition [21]. While the membrane charges will lower the effective pH at the surface [22], there will be a rather steep gradient to the higher pH in the medium. It is likely, therefore, that the proposed transition to an acid molten globule intermediate will need to be assisted by a component of deformation provided by the strongly polarised surface. This intermediate, although possessing the model molten globule characteristics of compactness, secondary structure and lack of persistent tertiary structure [23] and being stabilised by the same type of forces, may well differ in topology from that induced by the protonation of its carboxyl groups in a homogenous medium [24].

Further cases where an acid-induced molten globule

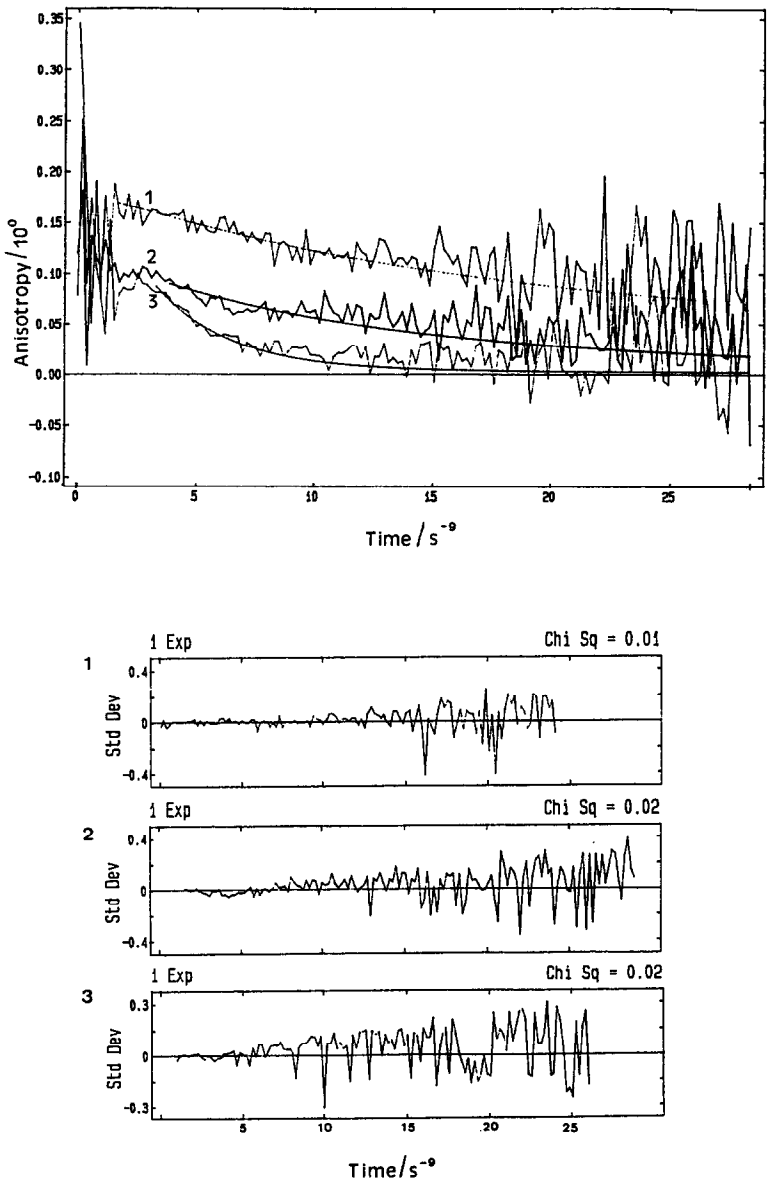

Fig. 3. The decay of tryptophan fluorescence anisotropy for TNF. Decays are for native (1), acid-denatured (2) and GdmCl-unfolded TNF (3). Single exponential fits to the data are shown for each case together with the residuals.

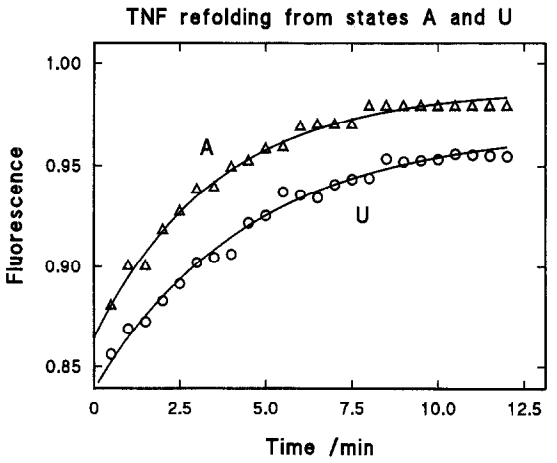

Fig. 4. The re-folding of acid-denatured (△) and GdmCl-unfolded TNF (○) monitored by the increase in the intrinsic protein fluorescence.

may be functionally important include the role of the acidic chaperone protein, DnaJ, in stabilising re-folding rhodanese in a molten globule form [25], and the finding that the acid molten globule of retinol binding protein is the form which releases retinol [26].

These examples suggest that the cell may have its means of generating molten globules in order to regulate protein function and that the molten globules concerned are not restricted to monomeric species.

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