

## Induction of $\alpha$ -Helix in the $\beta$ -Sheet Protein Tumor Necrosis Factor- $\alpha$ : Acid-Induced Denaturation

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**ABSTRACT:** Acid-induced unfolding of proteins often results in an intermediate structure, called the molten globule structure or “A” state, which retains at least partial secondary structure but lacks a rigid tertiary structure. Acid-induced unfolding has been studied extensively for  $\alpha$ -helical proteins, while few studies have been done on proteins containing only  $\beta$ -strands. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a trimer in which the individual subunits consist of antiparallel  $\beta$ -sheet, organized into a jellyroll  $\beta$ -sandwich. We have found previously [Narhi et al. (1996) *Biochemistry* 35, 11447–11453] that thermal denaturation of TNF- $\alpha$  results in an aggregate which contains a substantial amount of  $\alpha$ -helix and that the addition of trifluoroethanol induces  $\alpha$ -helix in both murine and human TNF- $\alpha$ . Here we show that acid also can induce  $\alpha$ -helix in these proteins. At acidic pH (below 4), both human and murine TNF- $\alpha$  convert to a monomeric form, as determined by sedimentation and diffusion constants obtained from sedimentation velocity experiments. The sedimentation coefficient indicated that this monomer was only slightly expanded relative to the native state. Near-UV circular dichroic (CD) analysis showed a loss of tertiary structure. These structural features coincide with the notion that the acid-induced structure of TNF- $\alpha$  is a molten globule. What is unique in this protein is that TNF- $\alpha$  acquires  $\alpha$ -helical structure, which is not present in the native structure as determined by both CD and Fourier transform infrared spectroscopy. Even more surprising is that TNF- $\alpha$  at pH 3.3 undergoes a very gradual noncooperative change in secondary structure upon heating, which results in an increase in  $\alpha$ -helical content. At pH 2.2 in the absence of salt, TNF- $\alpha$  shows considerable  $\alpha$ -helix, although heating does not change the spectrum. At pH 2.2, physiological salt decreases the amount of  $\alpha$ -helix at ambient temperature, and upon heating, we see the noncooperative increase in  $\alpha$ -helix as observed at pH 3.3 with low salt. The addition of salt at low pH induces reassociation but to a range of oligomers rather than a unique trimer structure. This acid-induced formation of an  $\alpha$ -helical monomer of TNF- $\alpha$  may be related to its known interaction with lipid bilayers.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ),<sup>1</sup> first observed by Carswell et al. (1975), plays an important role as a mediator of inflammation and the immune response (Ming et al., 1987; Sherry & Cerami, 1988). TNF- $\alpha$  and lymphotoxin, a related protein, have been shown to undergo an acid-induced change in conformation which results in an increase in surface hydrophobicity and a simultaneous increase in cytolytic activity, membrane insertion, and lipid vesicle binding (Yoshimura & Sone, 1987, 1990; Baldwin et al., 1988; Chang & Wisniewski, 1990). The inserted proteins appear to be capable of functioning as an ion channel (Kazan et al., 1992; Baldwin et al., 1995). These data have led to a hypothesis that the increased cytolytic activity seen at low pH is due to an acid-induced conformational change which allows the TNF- $\alpha$  to insert into lipid membranes and function as a Na<sup>+</sup> channel; this new conformation may also allow the TNF- $\alpha$  to penetrate directly into the cell, where neutral pH in the cytosol restores the original structure and activity (Kazan et al., 1992; Chang & Wisniewski, 1990).

TNF- $\alpha$  is a  $\beta$ -sheet protein which self-associates into a trimer (Arakawa & Yphantis, 1987; Davis et al., 1987; Narhi et al., 1987; Lewitt-Bentley et al., 1988). The crystal structure of human TNF- $\alpha$  (Jones et al., 1989; Eck & Sprang, 1989) and of lymphotoxin complexed with the p55 receptor (Banner et al., 1993) revealed that the individual TNF- $\alpha$  subunits consist almost entirely of antiparallel  $\beta$ -sheet, organized into a jellyroll  $\beta$ -sandwich, while the trimer is arranged about a 3-fold axis of symmetry.

It has recently been reported that human TNF- $\alpha$  is a helical trimer at acidic pH (Hlodan & Pain, 1994), and perhaps this trimer structure is what is incorporated into the membrane. However, it is very difficult to design a model that would allow for such a large change in the secondary structure while still maintaining the hydrophobic contact surface in the trimeric structure. In the preceding paper, we discussed the formation of  $\alpha$ -helix in TNF- $\alpha$  during thermal-induced denaturation. It is very interesting that acid can also induce an  $\alpha$ -helical conformation in a protein containing only  $\beta$ -sheet, such as TNF- $\alpha$ . Therefore, we have carried out a more detailed analysis of the acid-induced unfolding of TNF- $\alpha$ .

### MATERIALS AND METHODS

**TNF- $\alpha$  Preparation.** Recombinant human TNF- $\alpha$  was purified from *Escherichia coli* as described previously (Davis

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<sup>1</sup> Abbreviations: TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; CD, circular dichroism; FTIR, Fourier transform infrared; PBS, phosphate-buffered saline.

et al., 1987). The recombinant murine TNF- $\alpha$  was purified using the same procedure. Protein concentrations were determined from the absorbance at 280 nm, assuming an  $\epsilon_{280}^{0.1\%}$  of 1.26 for the murine molecule and 1.19 for the human molecule.

**Circular Dichroism.** The CD spectra were determined on a Jasco J-720 spectropolarimeter controlled by a DOS-based computer and Jasco software using cylindrical cuvettes with a path length of 0.02 cm for the far-UV region (250–190 nm) and a path length of 1 cm for the near-UV region (340–240 nm). Thermal denaturation was performed on the same instrument using a Peltier thermal control JTC-343 unit, a heating rate of 20 °C/h, and a rectangular cuvette with a path length of 0.1 cm. Mean residue ellipticity was calculated assuming a mean residue weight of 111.2.

**FTIR.** Murine TNF- $\alpha$  in PBS at pH 7 was concentrated to approximately 5 mg/mL with a Centricon-10 instrument (Amicon). Murine TNF- $\alpha$  in two different D<sub>2</sub>O buffers (PBS at pD 7.0 and 0.08% D<sub>3</sub>PO<sub>4</sub> and 140 mM KCl at pD 1.7) was prepared by repeated H<sub>2</sub>O–D<sub>2</sub>O exchanges with a Centricon instrument. The solution sample was placed between two CaCl<sub>2</sub> windows with a 6 mm spacer. FTIR spectra in the 1000–4000 cm<sup>−1</sup> range were collected on a Mattson Research Series Model 1000 with a MCT detector.

**Sedimentation Velocity.** Sedimentation velocity experiments were carried out in a Beckman XL-A apparatus using charcoal-Epon double sector cells at 60 000 rpm and 20 °C. Samples were dialyzed into the appropriate buffer, diluted to 60–150  $\mu$ g/mL, centrifuged at  $\sim$ 11000*g* for 2 min to remove large aggregates, and loaded into the centrifuge cell with dialysate in the reference sector. Concentration profiles were monitored by the absorbance at 230 nm. At the conclusion of the velocity run, the sample was removed and replaced by buffer after several fill/rinse cycles. Buffer/buffer baseline profiles were then recorded at 60 000 rpm to determine the zero offsets in the absorbance data.

The sedimentation velocity data were subsequently analyzed using the program SVEDBERG (Philo, 1994) or the Stafford *dc/dt* method (Stafford, 1992). Buffer densities and viscosities and corrections of sedimentation coefficients to standard conditions were calculated by the formulae in Laue et al. (1992).

## RESULTS

**CD Structure at acidic pH.** The effect of acid on the tertiary and secondary structure of human and murine TNF- $\alpha$  was first analyzed by CD. Figure 1A shows the near UV-CD spectra of human and murine TNF- $\alpha$  at pH 2.2 (0.085% phosphoric acid) and also of murine TNF- $\alpha$  at pH 2.2 plus 0.14 M KCl. Both forms of TNF- $\alpha$  show almost no signals at pH 2.2, regardless of the salt concentration, while they exhibit strong signals in PBS, indicating that they have lost all tertiary structure in acid.

The far-UV CD spectra of human TNF- $\alpha$  in PBS, at pH 3.3, and at pH 2.2 with and without salt are shown in Figure 1B, while the spectra of murine TNF- $\alpha$  in PBS, at pH 3.3 and pH 2.2 with no salt added, and at pH 2.2 in 0.14 M KCl are shown in Figure 1C. At neutral pH, in PBS, the spectra of the murine and human proteins are initially identical and are typical of  $\beta$ -sheet proteins of the TNF- $\alpha$  family. Fitting of the spectra by the method of Chang et al. (1978) indicates that they contain at least 50%  $\beta$ -sheet, consistent with the

crystal structure (Jones et al., 1988; Eck & Sprang, 1989). Lowering the pH results in a decrease in the ellipticity at both 208 and 222 nm in both molecules, as shown in Figure 1B,C. The ellipticity of the murine TNF- $\alpha$  at 208 nm increases from  $-3000$  at neutral pH (corresponding to 0%  $\alpha$ -helix) to  $-8000$  (14%) at pH 2.2 (Greenfield & Fasman, 1969). The human molecule appears to contain some random coil as well, with a minima at 198 nm. The addition of salt at pH 2.2 results in a decrease in the amount of  $\alpha$ -helix, as indicated by a decrease in ellipticity at 208 nm. The light scattering, observed as a high-tension voltage of the CD, is increased in these samples, indicating the formation of soluble aggregates. However, the solution remains clear, allowing for CD analysis. The spectra at pH 1.8 with and without salt are not shown but were very similar to those obtained at pH 2.2. These spectra of TNF- $\alpha$  at low pH are all very different from that of TNF- $\alpha$  in 6 M guanidine hydrochloride, where the TNF- $\alpha$  adopts a random coil configuration.

The human TNF- $\alpha$  appears to aggregate to a greater extent due to its increased hydrophobicity (Narhi et al., 1996). It thus appears that the structures of human and murine TNF- $\alpha$  are very similar, though the solubility is not. Both molecules are folded  $\beta$ -sheet proteins at neutral pH, and at acidic pH, they both exist as an intermediate or “A” state which contains  $\alpha$ -helix, and no tertiary structure. The amount of aggregate induced by acid is greater for the human TNF- $\alpha$ , resulting in some differences in CD spectra under these conditions; however, the induction of an  $\alpha$ -helical intermediate occurs in both systems.

**FTIR.** The second-derivative amide I' infrared spectrum of murine TNF- $\alpha$  in 0.085% D<sub>3</sub>PO<sub>4</sub> and 140 mM KCl at pD 1.8 is shown in Figure 2 and compared with the spectra of human and murine TNF- $\alpha$  in PBS at pD 7. The spectrum at low pD shows an IR band at 1653 cm<sup>−1</sup> characteristic of  $\alpha$ -helix. Curve-fitting results tabulated in Table 1 reveal that the murine TNF- $\alpha$  at pD 1.8 contains 12%  $\alpha$ -helix, consistent with the acid-induced formation of  $\alpha$ -helix seen in the CD spectra. This 1653 cm<sup>−1</sup> band is absent in the IR spectrum in PBS, consistent with there being no  $\alpha$ -helix in its structure at neutral pH. IR bands at 1625, 1636, and 1663 cm<sup>−1</sup> suggest that murine TNF- $\alpha$  in acidic buffer also contains a considerable amount of  $\beta$ -sheet as well as disordered structures. Extended antiparallel  $\beta$ -sheets also exist, though at relatively small amounts in murine TNF- $\alpha$  in this buffer, as indicated by the IR band at 1618 cm<sup>−1</sup>. These results corroborate the conclusions obtained from the CD results.

**Sedimentation Velocity.** The CD analysis described above demonstrates that the murine TNF- $\alpha$  has lost tertiary structure, which should affect the folding of the contact regions of the trimeric structure. Therefore, we have examined the state of association of the molten globular form of TNF- $\alpha$  at low pH by sedimentation velocity. It is well-known that under physiological conditions TNF- $\alpha$  is a trimer. Consistent with this, sedimentation velocity data for murine TNF- $\alpha$  in PBS (Figure 3A) give a sedimentation coefficient  $s_{20,w}$  of  $3.78 \pm 0.01$  S. We have also applied the program SVEDBERG (Philo, 1994) to these data to determine that the diffusion coefficient is  $D_{20,w} = (6.9 \pm 0.1) \times 10^7$  cm<sup>2</sup>/s. The ratio of  $s/D$  is proportional to molecular weight and in this case gives  $M_r = 51\,800 \pm 400$ , consistent with the sequence weight of 52 064 for a trimer.

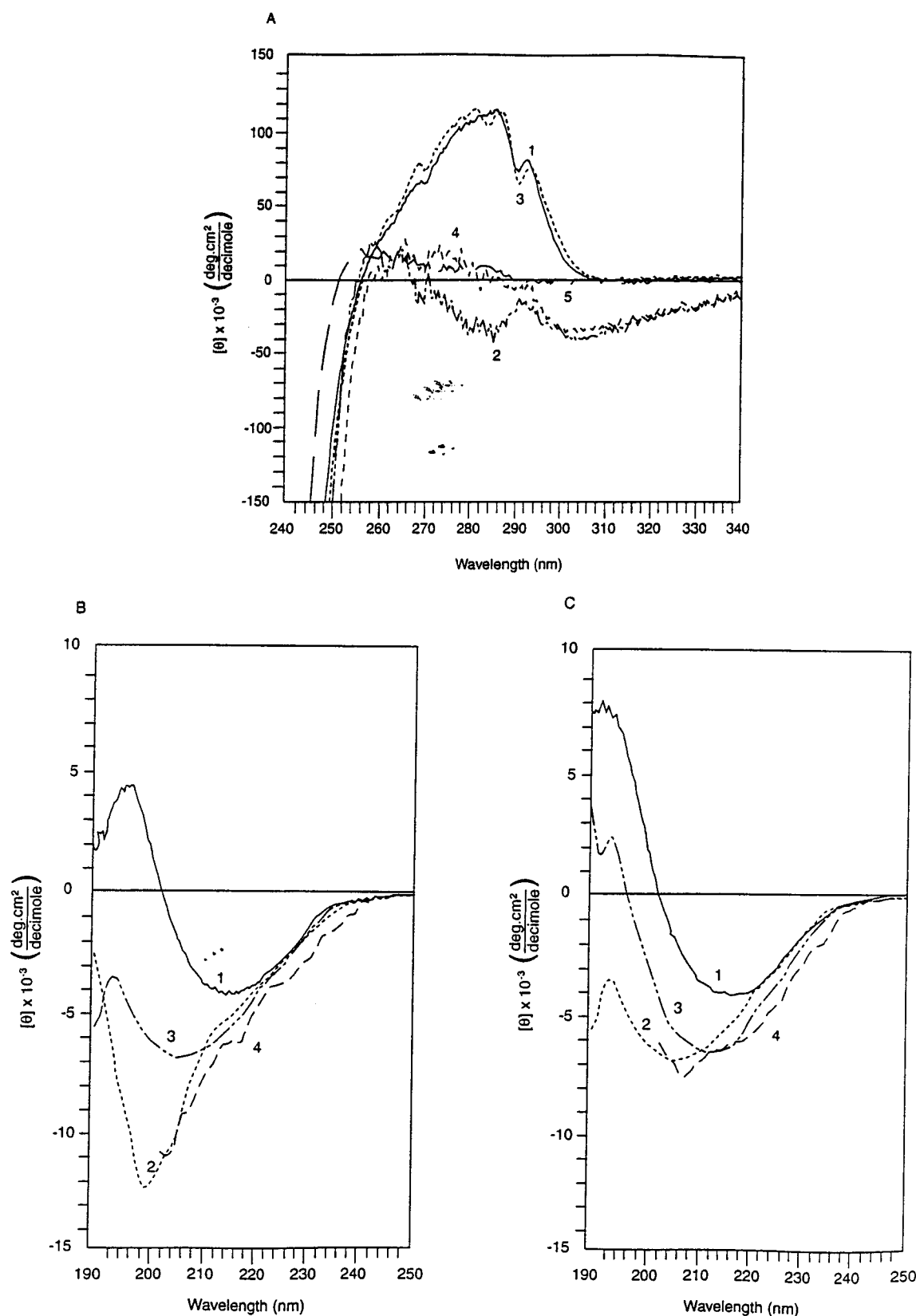


FIGURE 1: CD spectra of TNF- $\alpha$  at various pHs. (A) Near-UV CD spectra of human TNF- $\alpha$  in PBS (1, —), human TNF- $\alpha$  in 0.085% phosphoric acid at pH 2.2 (2, -·-·-), murine TNF- $\alpha$  in PBS (3, ···), murine TNF- $\alpha$  in 0.085% phosphoric acid at pH 2.2 (4, - - -), and murine TNF- $\alpha$  in 0.085% phosphoric acid at pH 2.2 and 140 mM KCl (5, -·-·-). (B) Far UV CD spectra of human TNF- $\alpha$  in several buffers: human TNF- $\alpha$  in PBS (1, —), human TNF- $\alpha$  in 0.085% phosphoric acid at pH 2.2 (2, ···), human TNF- $\alpha$  in 0.085% phosphoric acid at 0.14 M and pH 2.2 (3, -·-·-), and human TNF- $\alpha$  in 17.5 mM acetic acid pH 3.3 (4, - - -). (C) Far-UV CD spectra of murine TNF- $\alpha$  in several buffers: murine TNF- $\alpha$  in PBS (1, —), in 0.085% phosphoric acid at pH 2.2 (2, ···), in 0.085% phosphoric acid and 0.14 M KCl at pH 2.2 (3, -·-·-), and in 17.5 mM acetic acid at pH 3.3 (4, - - -).

In contrast, in 0.085% phosphoric acid (Figure 3B), the  $s_{20,w}$  drops to  $1.750 \pm 0.005$  S, and  $s/D$  implies  $M_r = 18\,000$

$\pm 1000$ , clearly indicating a monomeric state. Theory predicts that the sedimentation coefficient of a triangular

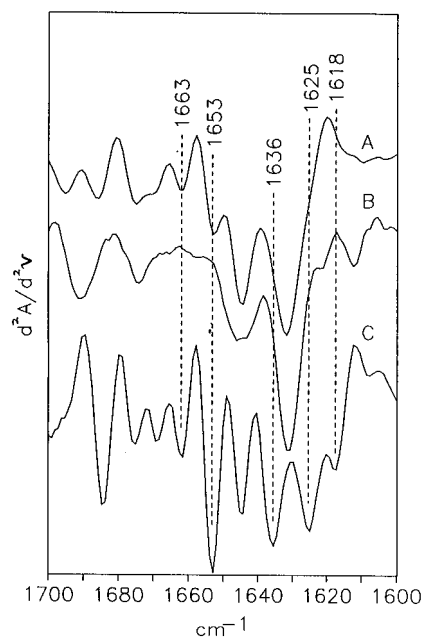


FIGURE 2: Second-derivative amide I' infrared spectra of (A) human and (B) murine TNF- $\alpha$  in PBS at pD 7 and murine TNF- $\alpha$  (C) at pD 1.8, both at 30 °C.

Table 1: Peak Positions, Relative Intensities, and Band Assignments of the Curve-Fitted Amide I' Components for Murine TNF- $\alpha$  at pD 1.8

$\nu$	A (%)	assignments
1617	7.0	$\beta$ -strand
1623	9.7	$\beta$ -strand
1630	14.6	$\beta$ -strand
1637	17.3	$\beta$ -strand
1645	15.4	disordered
1653	12.1	$\alpha$ -helix
1661	12.1	loops
1668	3.1	reverse turns
1674	6.5	reverse turns
1687	2.2	reverse turns

trimer will be 2.0 times that of a monomer if the subunits act as rigid spheres (van Holde, 1975), whereas the ratio here is 2.16. This indicates that the acidic monomer is somewhat less hydrodynamically compact than expected for a *native* monomer, consistent with its designation as a molten globule or A state. Thus, TNF- $\alpha$  is monomeric and less compact at low pH and low ionic strength. Similar results were obtained at pH 3.3. This is consistent with the FTIR and CD results described above.

This result appears to be quite different from that of Hlodan and Pain (1994), who reported that the acidic molten globule state is a trimer. Their conclusion was based largely on a measurement of sedimentation velocity in 0.1 M KCl/HCl at pH 1.6, for which they report  $s_{20,w} = 3.98$  S. On the basis that comparison of this value with sedimentation coefficients of other globular proteins implies a molecular weight of 56 000, they concluded that TNF- $\alpha$  is trimeric under these conditions. However, a comparison to a native trimer control clearly shows that this sedimentation coefficient is too high for a native trimer.

We find that addition of salts to acidic TNF- $\alpha$  has a profound effect on its state of association. In agreement with Hlodan and Pain, we also find an average  $s_{20,w} = \sim 4$  S in 0.1 M KCl/HCl at pH 1.6 if a simple analysis of the movement of the center of the boundary is used, but  $s$

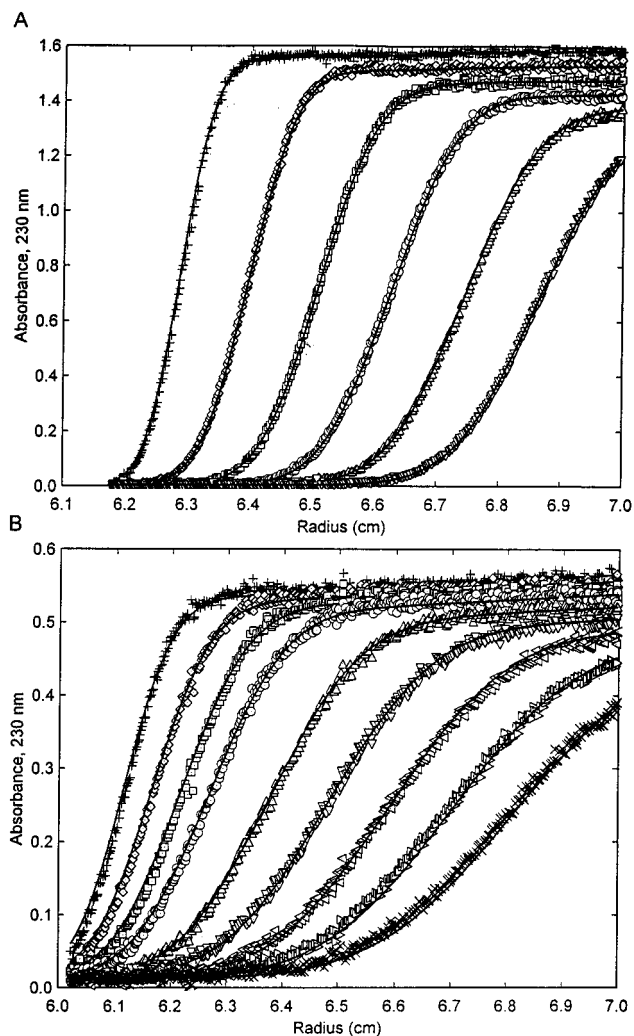


FIGURE 3: Sedimentation velocity data (points) and fitted curves (lines) for murine TNF- $\alpha$  at 60 000 rpm: native TNF- $\alpha$  trimer in PBS (A), scans starting at 15–134 min during the run; and acidic molten globule, 0.085% phosphoric acid (B), scans starting at 52–312 min.

increases to  $\sim 4.5$  S in 0.085% phosphoric acid and 140 mM KCl. Not only are these values higher than the value for the native trimer, but the shape of the boundary clearly indicates that under these conditions there is a distribution of species with different sedimentation coefficients.

We therefore analyzed the sedimentation velocity data using  $g(s^*)$  analysis (Stafford, 1992) to derive a distribution of sedimentation coefficients for these multiple species. The results of such analyses are shown in Figure 4, along with control data for the native trimer in PBS analyzed by the same technique, for both human and murine TNF- $\alpha$  and in both pH 1.6 KCl/HCl and 0.085% phosphoric acid and 140 mM KCl. The width of the distribution for the native trimer is due to diffusion and does not indicate a true distribution of sedimentation coefficients. The fact that the  $g(s^*)$  distributions for both human and murine TNF- $\alpha$  in acid and salt are much broader and shifted to higher  $s$  values clearly indicates that they consist of a distribution of species and that there is a considerable fraction of species much larger than the native trimer (since the distributions extend to at least 14 S, these samples contain oligomers of  $> 20$  monomers).

The small amplitude of the  $g(s^*)$  distributions at  $S$  values corresponding to the native trimer for the samples in acid

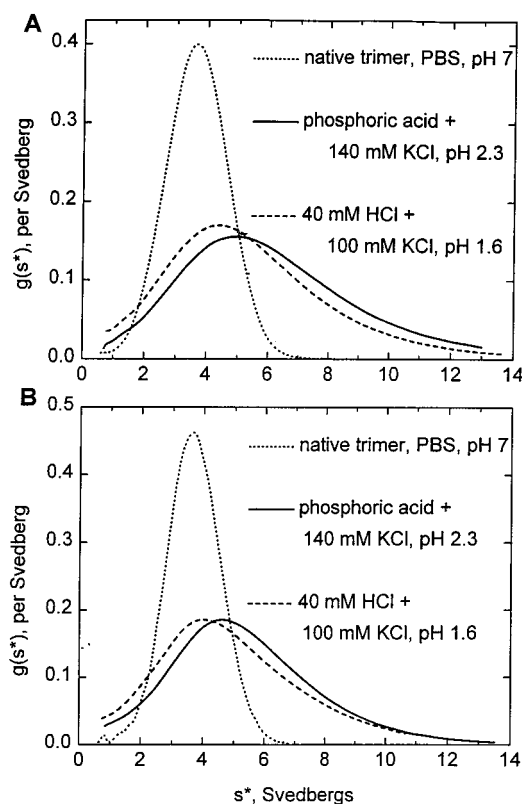


FIGURE 4: Sedimentation coefficient distributions for human TNF- $\alpha$  (A) and murine TNF- $\alpha$  (B) in PBS (dotted line), 0.085% phosphoric acid and 140 mM KCl (solid line), and 40 mM HCl and 140 mM KCl (dashed line). The distributions have been normalized so the total area under each curve is 1.0; thus, the values reflect the relative weight fraction at each sedimentation coefficient.

and salt indicates that these samples contain  $\leq 40\%$  trimer. This is also a significant amount of material sedimenting more slowly than the native trimer, indicating the presence of dimers and possibly monomers. We therefore conclude that salt induces association or aggregation of the acidic molten globule into a distribution of oligomers, not reassociation into a specific trimeric structure. The change in association state upon addition of salt, as well as small differences in the CD spectra, implies that TNF- $\alpha$  does not have a unique acidic molten globule state, but rather two or more conformations which meet the usual definitions of an A state. This is similar to the behavior of  $\beta$ -lactamase and several other proteins which can adopt at least two conformations at low pH, depending on the amount of salt present (Fink et al., 1994).

**Thermal Denaturation in Acid.** Figure 5 shows the effect of pH on the thermal denaturation of murine TNF- $\alpha$  in acid as determined by CD. The change in ellipticity at 215 nm (minimum for TNF- $\alpha$  at neutral pH) with temperature in different buffers is shown in Figure 5A, while Figure 5B shows the changes which are induced by heat throughout the spectrum. The thermal transition at pH 3.3 is not cooperative and occurs over a much wider temperature range than the thermal transition at neutral pH, consistent with the behavior of a molten globule. Further induction of  $\alpha$ -helix appears to occur at high temperatures, as indicated by a further decrease in the ellipticity at 208 nm in the 86 °C spectra from  $-7000$  to  $-10\,000$  [21%  $\alpha$ -helix using the Greenfield-Fasman equation (1969)] at pH 3.3. At pH 2.2 (and pH 1.8, not shown), there is essentially no further increase in  $\alpha$ -helical content with temperature. The unfolded

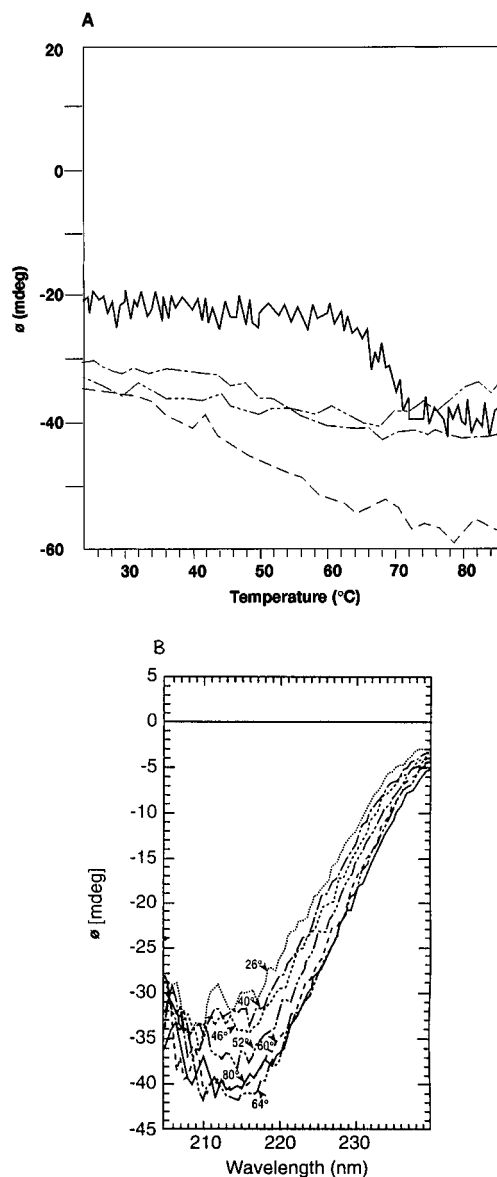


FIGURE 5: Thermal-induced denaturation of murine TNF- $\alpha$ . The change in ellipticity from 200 to 240 nm was monitored as a function of temperature. (A) Changes in ellipticity at 215 nm in PBS (—), in 17.5 mM acetic acid at pH 3.3 (---), in 0.085% H<sub>3</sub>PO<sub>3</sub> at pH 2.2 (···), and in 0.085% H<sub>3</sub>PO<sub>3</sub> and 150 mM NaCl at pH 2.2 (- · -) are plotted here. (B) Individual spectra obtained during melting of murine TNF- $\alpha$  at pH 3.3 in the transition region.

$\alpha$ -helical form remains soluble, but the unfolding is irreversible at every pH examined.

The effects of salt on thermal denaturation were also examined at low pH. At both pH 2.2 (Figure 5A) and 1.8 (data not shown), the addition of 150 mM NaCl results in a decrease in  $\alpha$ -helix, as demonstrated previously. Increasing the temperature under these conditions results in a noncooperative increase of  $\alpha$ -helix, as also shown in Figure 5A.

## DISCUSSION

Previously, we have observed the induction of  $\alpha$ -helix during the thermal unfolding of TNF- $\alpha$  at neutral pH (Narhi et al., 1996). In this paper, we have shown that acidic pH (below 4) also induces  $\alpha$ -helix in TNF- $\alpha$  (both human and murine). Although the acidic state has no defined tertiary structure and, in the absence of salt, it is dissociated into a monomer, it is relatively compact as demonstrated by its

sedimentation coefficient. Thermal unfolding of this acidic structure shows a very gradual change in secondary structure, indicating that it does not have cooperative interactions within the compact structure. These results indicate that TNF- $\alpha$  in acid assumes a molten globule or A state. The behavior of this molten globule-like structure is interesting in several respects.

This molten globule is unique in that it contains no  $\alpha$ -helix in the native state but acquires some  $\alpha$ -helix upon acid-induced unfolding. Although an acid-induced intermediate is an equilibrium structure, it has been suggested that similar structures occur as intermediates during the course of protein refolding (Kuwajima, 1989). If in fact this is true for TNF- $\alpha$ , the results described here imply that TNF- $\alpha$  refolding may occur via a compact  $\alpha$ -helical monomeric intermediate. Increasing the temperature even at pH 3.3 resulted in further induction of  $\alpha$ -helix as determined by CD. Although CD shows no tertiary structure at low pH and room temperature, there may be local or transient folding leading to the compact structure seen in the sedimentation velocity experiments done at pH 3.3 or 2.2. Induction of  $\alpha$ -helix at higher temperatures at pH 3.3 may reflect further loosening of the TNF- $\alpha$  structure and consequently a decrease in long range interactions throughout the protein, resulting in an increase in  $\alpha$ -helix. Since high temperatures should destabilize  $\alpha$ -helical structure, this loosening effect must overwhelm the heat destabilizing effect.

The formation of molten globule structures containing  $\alpha$ -helix has been seen in many proteins which contain  $\alpha$ -helix in the native structure. It can be induced by pH (Bullough et al., 1994; Fink et al., 1994), by TFE (Shiraki et al., 1995), and during refolding (Kuwajima et al., 1992). Induction of intermediates which contain even more helix than the native protein [the overshoot effect (Sugawara et al., 1991; Radford et al., 1992)] has also been reported. However, TNF- $\alpha$  is the only protein to date which has  $\alpha$ -helical structure in the molten globule but has no  $\alpha$ -helix in the native structure.

Another unusual property of acidic TNF- $\alpha$  is its response to the addition of salt. As noted above, addition of KCl to the acidic state causes a decrease in  $\alpha$ -helical content and inhibits further formation of  $\alpha$ -helix at higher temperatures. Both of these effects are probably linked to the fact that salt also causes the monomeric acidic state to associate into a range of oligomers/aggregates. Most likely, it is this aggregation which caused Hlodan and Pain (1994) to conclude that the acid-induced structure is trimeric. While it is true that the acid and salt state of TNF- $\alpha$  appears to result in some trimers, they are not the majority species. Hence, given that the acid-induced molten globules of most proteins tend to aggregate with the addition of high enough concentrations of salts (Arakawa et al., 1987; Artigues et al., 1994), it is difficult to say for certain whether acidic TNF- $\alpha$  can retain native-like and specific intersubunit interactions even though it has lost specific tertiary structure and added new  $\alpha$ -helical structure. It does appear, however, that TNF- $\alpha$  can adopt at least two different A states: a monomeric state in the absence of salt and an associated, less helical, state in the presence of salt.

Fink and co-workers (1994) have classified proteins into three categories on the basis of the effects of low pH or low pH plus salt on their structure. Type I proteins unfold to an acid-unfolded form (UA) which then refolds upon the addition of salt into a more compact A state; type II proteins

unfold in acid directly into the A state and are not affected by salt, and Type III proteins remain in an essentially native (N) state in acid.

The effect of salt can be explained as the addition of anions to a fully protonated protein resulting in the masking of some charged groups, decreasing the charge/charge repulsion, and increasing hydrophobic collapse. TNF- $\alpha$  at low pH behaves like a type II protein with the protonation of the molecule removing interactions necessary to maintain tertiary and quaternary structure and resulting in a compact monomeric molten globule A state. The secondary structure present, however, as mentioned above, is  $\alpha$ -helix, which is not present in the native molecule. The addition of anions and consequent increase in hydrophobic interactions in this case appears to result in oligomer formation, with perhaps some collapse to a more native-like species as well.

The partially unfolded forms of TNF- $\alpha$  we have observed at equilibrium under acidic conditions may be related to intermediates that occur during refolding at neutral pH. Kinetic studies by Hlodan and Pain (1995) are consistent with a sequence from unfolded TNF- $\alpha$ , through three different partially folded monomeric states, followed by assembly into a trimeric molten globule, and a final slow annealing into the native state. The present studies have shown the existence of both monomeric and oligomeric acidic molten globule states with slightly different secondary structure. These acidic structures, and the conformational changes upon association, may be similar to the structures involved in the monomer-trimer step during refolding. Hlodan and Pain (1995) also report the transient accumulation of a dimeric species during refolding, and indeed, we also see evidence for dimers under acid and salt conditions. This analogy breaks down, however, in that we find species larger than trimer that were not seen during refolding.

Under acid conditions, TNF- $\alpha$  has been shown to insert into membranes and liposomes and is capable of functioning as an ion channel (Yoshimura & Sone, 1987, 1990; Baldwin et al., 1988, 1996). The formation of  $\alpha$ -helix at this pH could facilitate some of these changes, as well as the change in surface hydrophobicity also reported (Chang & Wisniewski, 1990; Chang et al., 1988; Kazan et al., 1992). This might be physiologically relevant, as a pH of about 3.6 has been reported in the space between macrophages and their substrates (Silver et al., 1988). The  $\alpha$ -helical monomer might also be capable of passing through the cell membrane and into the cytosol itself, where the change in pH would result in a return to the  $\beta$ -sheet conformation, consistent with the model of Chang and Wisniewski (1990). It is also possible that upon interaction with membrane lipids the acidic molten globular structure may be altered to form a channel-like structure as observed by Baldwin et al. (1996).

The ability of a protein to assume different secondary structures depending on the presence or absence of long range interactions is also very interesting from the perspective of protein folding and is consistent with the thermal-induced denaturation of TNF- $\alpha$  reported in the previous paper (Narhi et al., 1996). Both long and short range interactions appear to be important during the refolding of BPTI (Ittah & Haas, 1995), and formation of  $\alpha$ -helix during the unfolding of the  $\beta$ -sheet protein epidermal growth factor has also been reported (Prestrelski et al., 1992; Narhi et al., 1992). TNF- $\alpha$  is a  $\beta$ -sheet protein which, as Shiraki and co-workers (1995)

found for  $\beta$ -lactoglobulin, might fold in a nonhierarchical fashion.

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