Early Aggregated States in the Folding of Interleukin-1 β

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Abstract. Kinetic data measured from folding of the protein interleukin-1 β fits best to three exponential phases when studied with tryptophan fluorescence but only two exponential phases when measured using other methods. The technique of ANS fluorescence was used to determine whether the additional phase observed in tryptophan fluorescence was also detected with ANS dye binding. Unlike tryptophan fluorescence, the ANS fluorescence was highly dependent on the concentration of protein present during the folding experiment. Experimental controls provide evidence that ANS binds to protein aggregates, present at higher concentrations and absent at lower concentrations. Protein concentration-dependent folding studies demonstrate that, at lower interleukin-1 β concentrations, tryptophan fluorescence kinetics can be fit adequately with a two exponential fit. This study indicates that (1) measured interleukin-1 β folding kinetics fit to a 2 phase model and (2) at higher protein concentrations, transient association of IL-1 β may result in a kinetic fit of 3 phases.

Key words: aggregation, 8-Anilino-1-naphthalenesulphonic acid, ANS, circular dichroism, inclusion bodies, interleukin-1 β , protein folding, stopped-flow kinetics, tryptophan fluorescence

1. Introduction

Protein aggregation has been increasingly studied in protein folding systems (Cleland, 1991; Goldberg, 1991; Georgiou, 1994; Haase-Pettingell, 1988). The kinetics of protein folding have been altered by transient aggregation prior to formation of the native state (Brems, 1988; Silow, 1999). In other cases, aggregation has been shown to be a stable by-product, often termed 'inclusion bodies,' which competes with the native protein during folding (Chan, 1996; De Bernardez-Clark, 1991). Amyloid-forming proteins have also been a subject of recent study, as the association of these proteins in neural fibrillar aggregates has been shown to correlate with neurological disorders (Lai, 1996; Cohen, 1994; Harper, 1997).

The cytokine interleukin-1 β (IL-1 β) is a protein which has changes in the observed kinetic rates of folding as a result of aggregation (Finke, 2000a), as well as irreversible formation of inclusion bodies *in vivo* (Chrnyk, 1993) and aggregates *in vitro* (Wetzel, 1994; Finke, 2000b). As a result, IL-1 β provides a versatile system in which to study protein folding under conditions with and without aggregation. A number of recent studies, both experimental and computational, have helped

to develop a well-defined mechanism for IL-1 β folding (Finke, 2000a; Heidary, 1997; Clementi, 2000). A well-defined kinetic model of IL-1 β folding is a crucial first step towards characterizing its less understood aggregation pathways.

Pulse-labelling experiments with IL-1 β were used to assess amide H-D exchange protection in main chain nitrogen atoms as folding progressed (Heidary, 1997; Varley, 1993). Results from both NMR and mass spectrometry analysis indicate that folding, measured by amide H-D exchange, occurred in two distinct steps. An early folding step, τ_2 , sequesters backbone atoms in the central β -strands 4–10 of the 12 total strands. The remaining strands 1–3 and 11–12 obtain protection in a second slower step, τ_1 , within 50 seconds. These results were reproduced in a complementary computational simulation, which added evidence supporting a 2 step IL-1 β folding mechanism (Clementi, 2000).

However, when real-time kinetics of IL-1 β folding were measured with tryptophan fluorescence and stopped-flow mixing (Finke, 2000a), three exponential folding phases were observed: $\tau_3 = 0.06$ sec (10% of signal), $\tau_2 = 0.25$ sec (50% of signal), and $\tau_1 = 57$ sec (40% of signal). The kinetic rates of τ_2 and τ_1 measured with fluorescence are in good agreement with the rates of τ_2 and τ_1 measured in the earlier pulse labelling experiments. The phase τ_3 has been detected by neither pulse labelling nor computational simulation.

Double-jump experiments have shown that the formation of native IL-1 β follows a single exponential process from an intermediate with relaxation time equal to τ_1 (Finke, 2000a). From these studies, the possible mechanisms of IL-1 β folding can be narrowed to the two possibilities shown in Figure 1.

The mechanism proposed in Figure 1A indicates that a single unfolded ensemble U folds at relaxation time τ_3 to an early intermediate ensemble I_1 , which folds at relaxation time τ_2 to a second intermediate ensemble I_2 , which folds to the native state N at relaxation time τ_1 . Intermediate ensemble I_2 corresponds to the intermediate observed in pulse-labelling studies with 44% of native amide H-D protection. Pulse-labeling studies do not detect the U to I_1 transition.

An alternative mechanism proposed in Figure 1B indicates that two slowly interconverting unfolded ensembles, U_1 and U_2 , exist prior to folding. They are distinguished kinetically by the relaxation time at which each ensemble folds to a single intermediate ensemble I , with U_1 folding at relaxation time τ_3 and U_2 folding at relaxation time τ_2 . Intermediate ensemble I folds to the native state N in a single pathway at relaxation time τ_1 . Intermediate ensemble I corresponds to the intermediate observed in pulse-labelling studies with 44% of native amide H-D protection. In this mechanism, pulse-labelling studies do not detect the U_1 to I transition.

It should be noted that the folding species indicated in the mechanisms proposed in Figure 1 are macroscopic populations with similar experimental properties. These simple pathway models would certainly become more complex if one was able to measure the microscopic properties of species within the macroscopic ensemble (Onuchic, 1997).

Figure 1. Two possible mechanisms of IL-1 β folding. A. Sequential progression of folded states, from a single unfolded ensemble U to an early intermediate ensemble I_1 , to a second intermediate ensemble I_2 , and finally to the native state N . B. Parallel paths from two distinct unfolded ensembles U_1 and U_2 to a single intermediate ensemble I . Native IL-1 β N folds from intermediate I through a single exponential process.

An important step towards the development a well-defined IL-1 β folding mechanism would be to disprove one of the two mechanisms, either Figures 1A or 1B. An obvious approach is to measure the folding kinetics of IL-1 β using additional probes to determine if the folding phase τ_3 can be further characterized.

In this study, we used two different probes, circular dichroism (CD) and 8-Anilino-1-naphthalenesulphonic acid (ANS) fluorescence, in conjunction with stopped-flow mixing, to measure the folding kinetics of IL-1 β . Changes in CD signal are sensitive to formation of α -helices and, to an extent, β -sheets from an unfolded random coil (Freifelder, 1982). CD is most sensitive to the native structure and fractionally sensitive to partially folded intermediates. Measurements by ANS fluorescence correlate with the binding of the ANS dye to hydrophobic surfaces on folding species (Semisotnov, 1987; Jones, 1994). ANS is most sensitive in the measurement of the formation and disappearance of folding intermediates during the folding of proteins. Together, CD and ANS fluorescence should complement the existing kinetic studies of IL-1 β folding. The number of exponential processes observed using CD and ANS fluorescence were determined by best fits to the kinetic data. Direct rate comparisons of processes detected in CD and ANS fluorescence kinetics were made to those detected by tryptophan fluorescence.

2. Methods

Wild-type IL-1 β and IL-1 β mutant K97I were cloned, expressed, and purified as described in Finke et al. (2000a). Purified K97I was dialyzed extensively into buffer containing 10 mM MES buffer containing 2 mM EDTA, 90 mM NaCl, and 1 mM β me at pH 6.5 for all experiments discussed.

Protein refolding was monitored prior to 20 seconds by tryptophan fluorescence, CD and ANS fluorescence with an Pi-Star (Applied Photophysics, London) stopped-flow unit with a pathlength of 0.1 cm. Stopped-flow tryptophan fluorescence was measured with excitation at 295 nm and emission collected through a >320 nm cut-off filter. Stopped-flow CD was measured at 230 nm. Stopped-flow ANS fluorescence was measured with excitation at 365 nm and emission collected through a >420 nm cut-off filter. Stopped-flow kinetic traces are the average of 10–20 data acquisitions.

Protein refolding monitored subsequently to 20 seconds by fluorescence at 230 nm used a Fluoromax-2 (Spex, NJ) with excitation/emission at 293/343 nm for tryptophan and 365/500 nm for ANS. Protein refolding monitored subsequently to 20 seconds by CD at 230 nm used an AVIV Model 202 Circular Dichroism Spectrometer with a cell pathlength of 1.0 cm. Manual-mixing kinetic traces are the average of 3 data acquisitions.

All refolding experiments were initiated by a 1:10 dilution of unfolded protein at 2.2 M Gdn-HCl into the indicated final Gdn-HCl and protein. Unless otherwise stated, the protein concentration was 10 μ M, the ANS concentration was 100 μ M (for ANS studies), and the temperature was 25 degC

Folding kinetics were fit to equation (1) using in-house software.

$$A(t) = \sum_{i=1}^n A_i e^{-\frac{t}{\tau_i}} + A_{\infty} \quad (1)$$

$A(t)$ = the measured signal at time t , n = the total number of observed processes measured by first-order fits, i = the i th of n processes, A_i = the amplitude of kinetic process i , A_{∞} = the equilibrium value of $A(t)$ at $t = \infty$, and τ_i = the observed relaxation time for process i . The values of n , A_i , A_{∞} , and τ_i were determined using the fit quality represented in the chi-squared (χ_r^2) values, the random dispersion of residuals, and the logical consistency of the generated fitting parameters. The value τ_i is an *observed* kinetic parameter and does not directly relate to the microscopic rate constant, k_i , for a kinetic process (Jennings, 1991).

3. Results

A comparison of relaxation times observed by tryptophan fluorescence and CD measurements of IL-1 β folding kinetics is shown in Figure 2.

Shown in Figure 2A are the CD and tryptophan fluorescence relaxation times measured for wild-type IL-1 β as a function of final guanidine hydrochloride (Gdn-

Figure 2. Folding kinetics of as measured by tryptophan fluorescence (open symbols) and circular dichroism (filled symbols) of folding phases τ_3 (triangle), τ_2 (square), and τ_1 (circle). A. Wild-type IL-1 β folding relaxation times at Gdn-HCl concentrations 0.2, 0.4, 0.6 and 0.8 M. B. K97I folding relaxation times at Gdn-HCl concentrations 0.2, 0.4, 0.6 and 0.8 M. Protein was initially unfolded at 110 μ M protein/2.2 M Gdn-HCl and rapidly diluted 1:10 to initiate folding at 10 μ M protein and the indicated Gdn-HCl concentrations. Relaxation times τ_i were obtained from fits of the kinetic data to equation 1.

HCl) concentration. Figure 2B indicates the CD and tryptophan fluorescence relaxation times measured for IL-1 β mutant K97I which, under these experimental conditions, has relaxation times different than those of wild-type. The relaxation times for K97I are also shown under varying final concentrations of Gdn-HCl.

In Figure 2A, the folding kinetics of the wild-type protein measured by tryptophan fluorescence fit best to $n = 3$ exponential processes, τ_3 (fast), τ_2 (medium), and τ_1 (slow). As expected for a typical protein folding reaction, the relaxation times increase at higher concentrations of final Gdn-HCl. The folding kinetics of the wild-type protein measured by CD fit best to $n = 2$ exponential processes, τ_2 (medium) and τ_1 (slow). The relaxation times for τ_2 and τ_1 are in good agreement between CD and tryptophan fluorescence. τ_3 is not observed in CD measured kinetics in wild-type IL-1 β .

The same value of $n = 3$ for tryptophan fluorescence and $n = 2$ for CD experiments are also measured in the folding kinetics of the mutant protein K97I in Figure 2B. The CD and tryptophan fluorescence relaxation times τ_2 and τ_1 for K97I are also in good agreement at all final Gdn-HCl concentrations, confirming that the two probes are consistent in an IL-1 β mutant protein. The relaxation time, τ_3 is not detected in K97I CD measurements. The fact that τ_3 is not observed by CD does not prove that it is not a real folding event. However, its absence in CD

kinetics implies a very small fractional contribution of the τ_3 amplitude, A_3 , to the CD signal.

Since τ_3 was not observed in CD, ANS fluorescence was used to determine if τ_3 could be characterized. An initial study, similar to that used in Figure 2, was performed using ANS fluorescence as a probe. However, the values of n and τ_i measured by ANS were highly contradictory with pulse-labelling, tryptophan fluorescence, and CD (data not shown). An experimental control revealed that ANS fluorescence was highly dependent on the concentration of IL-1 β in the folding reaction and therefore not representative of protein folding. The effect of IL-1 β concentration on *normalized* stopped flow kinetic data is shown in Figure 3.

Figure 3 shows ANS-fluorescence kinetic signal at varying IL-1 β concentrations and after normalization with equation (2).

$$N(t)_{X\mu M} = A(t)_{X\mu M} \frac{10\mu M}{X\mu M} \quad (2)$$

$N(t)_{X\mu M}$ is the normalized kinetic signal at each IL-1 β concentration X and $A(t)_{X\mu M}$ is the raw kinetic signal at each IL-1 β concentration X . The normalized amplitude, $N(t)_{X\mu M}$, is calculated using 10 μ M IL-1 β as the normalization reference.

ANS may bind preferentially to monomeric intermediate or native states over unfolded states, thereby generating ANS-fluorescence changes as these states populate during IL-1 β folding. ANS-fluorescence resulting from binding to these states should be linear with the concentration of protein. Thus, if only monomeric states exist during folding, equation (2) should be able to normalize all ANS kinetic traces in Figure 3A to an amplitude of approximately 1.0. However, as indicated in Figure 3A, the normalized amplitude decreases rapidly from 1.0 to -0.2 as the IL-1 β concentration is decreased from 20 μ M to 2 μ M.

This dramatic decrease in signal can be explained by one of two possibilities. Hypothesis A is that ANS binds preferentially to IL-1 β aggregates and that these aggregates are less populated at lower IL-1 β concentrations. Hypothesis B is that binding between IL-1 β monomers and ANS dye itself is thermodynamically unfavorable at decreased protein concentration. To test hypothesis B, the IL-1 β concentration was held constant during the folding reaction at 10 μ M and the ANS concentration was decreased. Each kinetic curve was normalized using equation (3).

$$N(t)_{Y\mu M} = A(t)_{Y\mu M} \frac{100\mu M}{Y\mu M} \quad (3)$$

$N(t)_{Y\mu M}$ is the normalized kinetic signal at each ANS concentration Y and $A(t)_{Y\mu M}$ is the raw kinetic signal at each ANS concentration Y . The normalized amplitude, $N(t)_{Y\mu M}$, is calculated using 100 μ M ANS as the normalization reference.

Figure 3. Refolding of IL-1 β at 0.4 M Gdn-HCl in the presence of ANS. A. Normalized refolding kinetic traces, $N(t)_{X\mu M}$, of ANS fluorescence from 100 μ M ANS and varying concentrations of IL-1 β . The $N(t)_{X\mu M}$ traces in Figure 3A have been normalized using equation 2. B. Normalized refolding kinetic traces, $N(t)_{Y\mu M}$, of ANS fluorescence from 10 μ M IL-1 β and varying concentrations of ANS. The $N(t)_{Y\mu M}$ traces in Figure 3B have been normalized using equation 3. Refolding was performed by a 1:10 dilution of initially unfolded protein at 2.2 M to 0.4 M Gdn-HCl.

If the ANS-fluorescence decrease at low IL-1 β concentrations in Figure 3A results from hypothesis B, one would expect a ANS-fluorescence decrease if protein concentration is held constant and the ANS concentration is decreased. However, as indicated in Figure 3B, the normalized kinetic signal remains relatively constant as the ANS concentration is varied and the IL-1 β concentration is held constant at 10 μ M. Thus, the decrease in normalized ANS signal at lower IL-1 β concentrations is the likely result the reduced population of ANS-sensitive IL-1 β aggregates at those concentrations.

To further test whether IL-1 β aggregation results in increased ANS fluorescence during folding, the kinetic rates and amplitudes of ANS fluorescence was measured at increased IL-1 β concentrations (Kiefhaber, 1991). Smaller relaxation times and larger amplitudes of ANS fluorescence signals at higher IL-1 β concentrations would support the premise of ANS-detected aggregation. Further support would also be provided by a smaller relaxation time and larger amplitude in the IL-1 β mutant K97I, which has been shown to have a much greater propensity for aggregation during folding (Finke, 2000a).

In addition to wild-type IL-1 β , the experiment in Figure 3A was also performed on K97I (data not shown). To qualitatively measure the kinetics of ANS-detected aggregation in wild-type IL-1 β and K97I, the normalized curves from Figure 3A were fit using equation (4).

$$A(t) = A_{ANS}e^{-\frac{t}{\tau_{ANS}}} + A_{\infty} \quad (4)$$

Aggregation, unlike folding, cannot be rigorously characterized by fitting a sum of n exponentials in equation (1). However, aggregation rate changes between multiple aggregation kinetic measurements, acquired in a controlled experiment, can be deduced by comparison of the time constant (τ_{ANS}) fit in equation (4) (Zettlmeissl, 1979). The amplitude of the fit (A_{ANS}) can also be used as a parameter to deduce changes in the amount of aggregation produced.

Figure 4A shows the change in amplitude A_{ANS} fit from the normalized ANS curves for wild-type IL-1 β and K97I at various protein concentrations. It clearly shows a decrease in signal at low concentrations – in fact, a negative amplitude at 2 μ M. The increased amplitude of K97I over wild-type ANS signal is evidence of ANS binding to aggregates. Figure 4B shows the change in relaxation time (τ_{ANS}) fit from the normalized ANS curves for wild-type IL-1 β (Figure 3A) and K97I at various protein concentrations. Both wild-type and K97I show a decrease in τ_{ANS} (i.e. increase in rate) at increasing concentrations, providing further evidence supporting ANS binding to aggregates.

Interestingly, the relaxation time fit from ANS kinetics (τ_{ANS}) is similar to tryptophan-fluorescence detected relaxation times τ_3 and τ_2 . It is possible that aggregation, if detected with ANS fluorescence, may also be detected by tryptophan fluorescence, even though pulse-labelling or circular dichroism appear to be insensitive to association events. When kinetic experiments measure the folding at high concentrations of IL-1 β , formation of aggregates may occur and add an additional signal contribution to the measured tryptophan fluorescence kinetics. Thus, kinetics at high IL-1 β concentrations may appear more complex than at low concentrations, such that two exponentials, τ_3 and τ_2 , are required to fit the kinetic data instead of one. If formation of aggregates during IL-1 β folding results in a two exponential fit instead of one, folding IL-1 β at lower concentrations of IL-1 β should reduce aggregation. Also, at low concentrations, one would expect folding kinetics to be adequately fit by a single exponential process. In the case of IL-1 β , fits of early folding kinetic data (< 5 seconds) measured by tryptophan fluorescence should

Figure 4. Kinetic refolding parameters A_{ANS} and τ_{ANS} from fits of normalized kinetic ANS fluorescence traces using equation 4. A. A_{ANS} as a function of IL-1 β concentration for wild-type ($\circ$) and K97I ($\bullet$). B. τ_{ANS} as a function of IL-1 β concentration for wild-type ($\circ$) and K97I ($\bullet$).

fit to a single exponential process at low IL-1 β concentrations. The slowest IL-1 β folding phase, τ_1 (57 seconds), is two orders of magnitude slower than τ_{ANS} (0.2 seconds) and would not be perturbed by the ANS-detected aggregation.

Aggregation, as measured by ANS, does not seem to be present at very low protein concentrations ($< 6 \mu$ M). Thus, fits of tryptophan fluorescence kinetic data at low concentrations should only require $n = 2$ exponentials, τ_2 and τ_1 , to

accurately fit the curve. To confirm this, tryptophan fluorescence during folding was examined at decreasing WT IL-1 β protein concentrations. It is observed that fits of the tryptophan fluorescence kinetic data at lower IL-1 β concentrations do not require inclusion of the τ_3 phase (data not shown). This observation suggests that fitting IL-1 β kinetics with three phases, τ_3 , τ_2 and τ_1 , instead of two, τ_2 and τ_1 , is not necessary at lower concentrations of IL-1 β .

4. Discussion

The results of these experiments have indicated that aggregation events during folding can potentially be experimentally mistaken as folding events. The effect of aggregation on folding kinetics has been observed in a number of previous studies (Silow, 1999; Brems, 1988). This is not due to any fault in the methods of the experiment but due to an alternative reaction coordinate which becomes available at higher protein concentrations (Oliveberg, 1998). It is necessary in protein folding studies to determine whether these aggregation pathways exist and, if so, identify the effects they have on the folding parameters.

The folding of a number of proteins has been studied with ANS. In many cases, it has proven to be a useful tool to study monomeric intermediates in a folding pathway (Jones, 1994; Semisotnov, 1987). However, as shown by the large increase of the normalized ANS signal between 2 and 20 μ M IL-1 β in Figure 3A, ANS is clearly much more sensitive to IL-1 β aggregates than IL-1 β monomeric folding intermediates. For this reason, any IL-1 β folding mechanisms based upon kinetic studies using ANS fluorescence need to be reconsidered (Varley, 1993).

The present study adds an important contribution to previous work on IL-1 β folding and aggregation. Earlier studies detected aggregation in IL-1 β using light scattering methods (Finke, 2000a; Chrnyk, 1993). In these studies, the aggregation was characterized as large insoluble oligomers and resulted in an irreversible loss of soluble protein (Finke, 2000b). The aggregation detected with ANS in this study differs from that detected previously with light scattering in that it is observed at concentrations where no loss of soluble protein occurs. Thus, these aggregates must be (1) small, since they are not detected with light scattering, and (2) reversible, since they feed back into the folding pathway to form native IL-1 β monomers.

The observation of reversible aggregates supports a nucleation model of IL-1 β aggregation during folding (Finke, 2000a, b). Detection of small reversible aggregates with ANS in the absence of large irreversible aggregates, detected with light scattering, provides strong evidence of a nucleation mechanism (Harper, 1997).

By combining previous studies of IL-1 β folding and aggregation with the present study, a revised mechanism of IL-1 β folding and aggregation can be proposed. This proposal is shown in Figure 5. The monomeric folding states U , U_C , I , and N are illustrated along the top while aggregated states are indicated underneath. Folding and aggregation states detected by ANS fluorescence are shown within the circle.

Figure 5. Revised mechanism of IL-1 β folding and aggregation. Early in the folding process ($\tau_{burst} < 1$ msec), the unfolded/denatured protein ensemble U collapses to a more compact unstructured ensemble U_C . On a measurable timescale, ($\tau_2 = 0.25$ sec), intermediate ensemble I is formed sequentially from U/U_C . On a slower timescale, ($\tau_1 = 57$ sec), native IL-1 β N is formed sequentially from intermediate I . Aggregation initiates from the U/U_C ensemble, on a timescale of approximately $\tau_{ANS} = 200$ msec, to form small unstable Non-Committed Aggregates. If the protein concentration is high enough to thermodynamically drive the aggregation to sizes larger than the Nucleus, Committed Aggregates will populate irreversibly. The relaxation time of the kinetic formation of these Committed Aggregates, under native-like conditions, has been measured at $\tau_{CA} = 55$ sec. Transitions between species U , I , and N are measurable by tryptophan fluorescence, circular dichroism, and pulse-labelling. Transitions between species U , U_C , and Non-Committed Aggregates are detected by ANS binding/fluorescence. Formation of the Nucleus and Committed Aggregates are detectable by light scattering.

In Figure 5, folding initiates from the completely unfolded ensemble, U . This ensemble is proposed to undergo a rapid (μ sec) collapse to a more compact, yet unstructured, ensemble. This proposal is based on a small ANS signal ‘burst’ in the dead-time (< 1 msec) of the stopped-flow instrument which does not decrease with protein concentration (Figure 3A). This burst state is not detected in either pulse-labelling (Heidary, 1997), tryptophan fluorescence (Finke, 2000a), or CD and is therefore assumed to represent a minimal structural increase from the initial unfolded ensemble. U_C folds with relaxation time τ_2 to a partially structured intermediate ensemble I , comprised of microscopic states which share the macroscopic property of approximately 50% native signal by most structural probes. Lastly, intermediate ensemble I folds with relaxation time τ_1 to the native state.

Aggregation has been shown to initiate from a species present in the dead time of stopped-flow mixing (Finke, 2000a). Whether this species corresponds to the ensemble U , U_C , or both, will be the subject of future study. In Figure 5, aggregation initiates through an unstable ensemble of aggregate(s) which, if no further aggregation occurs, will disassociate and refold properly. We propose that these 'Non-Committed Aggregates' are the species detected using ANS fluorescence. If the protein concentration during folding is high enough, aggregation will be favored to form a nucleus. The nucleus is defined as the aggregate of highest energy and least stability (Harper, 1997). Sequential binding of monomers to aggregates before nucleus formation becomes less and less energetically favorable. Additional binding of monomers to the nucleus becomes more and more energetically favorable. Thus, in the mechanism proposed in Figure 5, formation of species larger than the nucleus 'commits' these species to essentially irreversible aggregated states. The formation of these 'Committed Aggregates' has been measured with an apparent first order relaxation time of $\tau_{CA} = 55$ sec and are also detectable with light scattering (Finke, 2000b).

The results of the CD and ANS studies complement well the previous work on Il-1 β . Specifically, the results of this study have supported a single folding mechanism, an essential step towards more complex protein folding analyses and aggregation studies. The simple model proposed in Figure 5 will provide the stage for a number of interesting future experiments.

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