Autocatalyzed Protein Folding†

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ABSTRACT: Proline isomerization, an intrinsically slow process, kinetically traps intermediates in slow protein folding reactions. Thus, enzymes that catalyze proline isomerization (prolyl isomerases) often catalyze protein folding. We have investigated the folding kinetics of FKBP, a prolyl isomerase. The main conclusion is that FKBP catalyzes its own folding. Altogether, the FKBP refolding kinetics are resolved into three exponential phases: a fast phase, τ_3; an intermediate phase, τ_2; and a slow phase, τ_1. Unfolding occurs in a single phase, the unfolding branch of phase τ_2. In the presence of native FKBP, both the intermediate (τ_2) and slow (τ_1) phases are faster, suggesting that folding phases τ_1 and τ_2 involve proline *cis*−*trans* isomerization. In the absence of added native FKBP, autocatalytic folding of FKBP is detected. For refolding starting with all the FKBP unfolded initially, the slowest folding phase (τ_1) is almost 2-fold faster at a final concentration of 14 μM FKBP than at 2 μM FKBP, suggesting that catalytically active FKBP formed in the fast (τ_3) or intermediate (τ_2) folding phases catalyzes the slow folding phase (τ_1). Moreover, autocatalysis of folding is inhibited by FK506, an inhibitor of the FKBP prolyl isomerase activity. The results show that the slow phase in FKBP folding is an autocatalyzed formation of native FKBP from kinetically trapped species with non-native proline isomers. While the magnitude of the catalytic effects reported here are modest, FKBP folding may provide a prototype for autocatalysis of kinetically trapped macromolecular conformational changes in other systems.

FK506 binding protein (FKBP)[1] is a small globular protein of molecular weight 11 750 made up of five β-strands, an α-helix, and three loops (Van Duyne et al., 1991). It belongs to a family of prolyl isomerases that are efficient catalysts of proline *cis*−*trans* isomerization in short, unstructured peptides (Harrison & Stein, 1990). The two most studied prolyl isomerases are FKBP-12 and human cyclophilin A (CyP). Immunosuppression results from the binding of immunosuppressants by the immunophilins (FKBP or CyP) and subsequent interaction with calcium and the calmodulin-dependent phosphatase, calcineurin, which leads to inhibition of specific signal transduction pathways needed for T-lymphocyte activation (Schreiber, 1991). CyP is known to catalyze protein folding *in vitro* and *in vivo* (Schmid, 1995), and FKBP from *Neurospora crassa* has been shown to catalyze *in vitro* protein folding (Tropschug et al., 1990). Using unstructured peptides as substrates, CyP catalysis of *cis*−*trans* isomerization *in vitro* is more efficient and less dependent on sequence than FKBP catalysis (Harrison & Stein, 1990).

Here we describe studies of the equilibrium and kinetic properties of the Gdn·HCl-induced folding/unfolding of FKBP. The Gdn·HCl-induced equilibrium unfolding transition is fully reversible and compatible with a two-state unfolding transition. Urea-induced equilibrium unfolding of FKBP has been reported previously to be fully reversible (Egan et al., 1993). The kinetics of refolding from Gdn·HCl are similar to those of many other small globular proteins. Both fast and slow folding phases are detected, while unfolding occurs in a single fast kinetic phase. Catalysis of FKBP slow folding by prolyl isomerases (FKBP and/or CyP) indicates that the slower time scale arises from coupling of folding to an intrinsically slow process, proline *cis*−*trans* isomerization. A surprising feature of slow folding is that the rate increases as the final FKBP concentration increases, suggesting that FKBP catalyzes its own slow folding.[2]

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[1] Abbreviations: FKBP (or FKBP-12), FK binding protein, an FK 506 binding protein with peptidyl−prolyl *cis*−*trans* isomerase activity; FK 506, an immunosuppressant drug that binds to FKBP and inhibits the peptidyl−prolyl *cis*−*trans* isomerase activity; CyP, a cyclosporin A binding protein with peptidyl−prolyl *cis*−*trans* isomerase activity, also known as human cyclophilin (hCyP); PPI, a peptidyl−prolyl *cis*−*trans* isomerase, e.g., FKBP or CyP; CsA, cyclosporin A; Suc-AAPF-pNA, tetrapeptide substrate used in the determination of the specific activity of FKBP and CyP (succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide); DTT, dithiothreitol; β-ME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TEM buffer, 0.02 M Tris buffer, pH 8.0, 1 mM EDTA, 1 mM β-ME; k_{cat}/K_m, catalytic efficiency of CyP or FKBP; $(k_{cat}/K_m)_{pep}$, catalytic efficiency of CyP or FKBP toward the peptide substrate; $(k_{cat}/K_m)_{prot}$, catalytic efficiency of CyP or FKBP toward the protein substrate (FKBP); $(k_{cat}/K_m)_{rel}$, catalytic efficiency of CyP or FKBP toward the protein substrate relative to the peptide substrate.

[2] A recent report of refolding from urea solutions also concludes that FKBP folding is autocatalytic (Scholz et al., 1996).

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The magnitude of the FKBP catalytic effects is modest. Nevertheless, FKBP provides an intriguing example of a new class of macromolecular autoactivation reaction in which an isomerase enhances the rate of formation of additional isomerase activity. The situation is not unlike that of aspects of zymogen activation in which a protease catalytically converts its own proenzyme to the functional protease (Ikemura & Inouye, 1988). More generally, FKBP is a catalyst of slow folding or conformational reactions necessary for its own folding. Interestingly, the underlying features of FKBP-(auto)catalyzed folding are much the same as those proposed for formation of infectious scrapie prions, PrP^{Sc}, from kinetically trapped folding intermediates, PrP^{C}: i.e., PrP^{Sc} enhances the rate of conversion of (noninfectious) PrP^{C} to PrP^{Sc} (Huang et al., 1996).

MATERIALS AND METHODS

Protein Isolation and Characterization. FK506 binding protein (FKBP) was purified from FKBP-pHN1/XA90 *Escherichia coli* cells. The FKBP-pHN1 plasmid and the XA90 *E. coli* host were obtained as gifts from Drs. Robert Standaert, Stuart L. Schreiber, and Gregory L. Verdine (Standaert et al., 1990). FKBP was also purified from *E. coli* containing the CKS-rhFKBP fusion construct according to the published procedures (Edalji et al., 1992). Purified FKBP was stored frozen at −70 °C or lyophilized from HEPES buffer and stored at 4 °C. Stock solutions were prepared from the lyophilized enzyme by dissolving the protein in deionized distilled water, dialyzing exhaustively against 0.02 M Tris buffer, pH 8.0, 1 mM EDTA, and 1 mM β-ME (TEM buffer), and sterile filtering the solution. Concentrated stocks thus obtained were stored at 4 °C. Stocks of native FKBP were prepared by dilution with buffer, and unfolded stocks were prepared by dilution into buffer containing a high concentration of denaturant (1.8−3 M Gdn·HCl). Protein concentration was estimated using a molar extinction coefficient at 280 nm of $\epsilon_{280} = 9860\ M^{-1}\ cm^{-1}$ calculated by the method of Gill and von Hippel (1989).

Human cyclophilin (CyP) was purified from pHNJ/XA90 *E. coli* cells that overexpress human CyP. The CyP overexpression *E. coli* strain pHNJ/XA90 was a gift of Drs. Christopher Walsh and Jun Liu. CyP was purified in the presence of reducing agent, β-ME or DTT (Liu et al., 1990), chromatographed by elution from CM-Sephadex cation-exchange resin with a 0−0.25 M sodium chloride gradient in TEM buffer. Purified CyP was stored at 4 °C and used within 2 months or stored at −70 °C and used within 6 months. Enzyme concentration was determined by Bradford assay and/or using the molar extinction coefficient of $\epsilon_{280} = 8730\ M^{-1}\ cm^{-1}$ calculated on the basis of aromatic amino acid content (Gill & von Hippel, 1989). The effect of Gdn·HCl on the specific activity of CyP was determined by conducting the enzyme assays, under the same conditions as for refolding of FKBP.

Equilibrium Unfolding. To be sure that equilibrium had been attained, samples were prepared by refolding from a fully unfolded stock solution and unfolding from a fully folded stock solution. An unfolded stock solution containing about 100 μM FKBP in 2 M Gdn·HCl was allowed to equilibrate at 20 °C for at least 1 h. The unfolded FKBP was diluted into TEM buffer containing sufficient Gdn·HCl to give a refolded protein solution at each desired final denaturant concentration. The folded protein stock solution was prepared in TEM buffer and diluted into buffer containing Gdn·HCl. Solutions thus prepared, after thorough mixing of each solution, were equilibrated for 1−24 h at 20 °C before determining the intrinsic fluorescence of the solution. The final concentration of FKBP was 2 μM. All buffers and Gdn·HCl stocks were prepared in thoroughly washed glassware to prevent fluorescent contamination. Blank solutions were prepared containing buffer and Gdn·HCl, and fluorescence standards were prepared containing buffer, Gdn·HCl, and *N*-acetyltryptophanamide (NATA). Samples, blanks, and standards were all prepared in Falcon or Corning tubes as these were found to be largely free of fluorescent contaminants. Fluorescence measurements were made at 20 °C in an SLM Aminco SPF 500C spectrofluorometer with excitation at 285 nm (5 nm bandwidth) and fluorescence detection at 350 nm (7.5 nm bandwidth). The excitation wavelength of 285 nm was chosen to excite both tyrosine and tryptophan in FKBP (FKBP contains three Tyr and one Trp) so that the fluorescence changes would probe the environment of several regions of the protein simultaneously. The fluorescence of the blank solutions was subtracted from the fluorescence of FKBP samples and standard (NATA) samples before calculating the relative fluorescence intensity. The relative fluorescence was taken as the ratio of the fluorescence of the FKBP solution to that of an equal molar solution of NATA.

Kinetic Measurements. The procedures for measuring the rates of folding of FKBP in the presence and absence of CyP are similar to those described previously for CyP-catalyzed folding of cytochrome *c* (Veeraraghavan & Nall, 1994). Stopped-flow mixing experiments were carried out using a Bio Logic SFM-3 stopped-flow spectrofluorometer with an FC-20 Suprasil observation cuvette. The flow rate/observation cuvette combination used for the stopped-flow experiments resulted in mixing times of 4−7 ms. Fluorescence excitation was at 285 nm (15 nm bandwidth) with observation through a 366 nm bandpass filter (54 nm bandwidth). Manual mixing experiments were carried out using an SLM Aminco SPF 500C spectrofluorometer with 285 nm excitation (5 nm bandwidth) and detection at 350 nm (7.5 nm bandwidth). As a test of whether there were differences in the refolding kinetics for excitation of both tryosines and tryptophans compared to selective excitation of the single tryptophan, a manual mixing experiment was carried out with 295 excitation (5 nm bandwidth) and detection at 350 nm (7.5 nm bandwidth). Refolding monitored with 295 nm excitation gave essentially the same refolding rates and relative amplitudes as 285 nm excitation. Manual mixing deadtimes are typically 5 s. For both stopped-flow and manual mixing experiments, digitized kinetic traces of 1000 data points were analyzed with the Bio-Kine software supplied with the Bio Logic stopped flow (Molecular Kinetics, Inc., Pullman, WA). Apparent first-order rate constants, k_i, and the corresponding amplitudes, ΔF_i, were obtained by nonlinear least squares fits of the data to the equation:

$$\Delta F(t) = \sum_i \Delta F_i \exp(-k_i t) \qquad (1)$$

where $\Delta F(t)$ is the difference between the fluorescence at t and the final fluorescence at equilibrium. ΔF_i and k_i are the fluorescence amplitude and apparent rate constant as-

sociated with kinetic phase *i*. In some cases rates are expressed as time constants, τ_i, which are the inverse of the apparent rate constants ($\tau_i \equiv 1/k_i$). Generally, it is possible to separate rate processes differing by more than 3-fold in rate into discrete kinetic phases. The apparent rate constants k_1 and k_2 differ by 4−9-fold for most final Gdn·HCl concentrations used for refolding. However, near 0.5 M Gdn·HCl k_1 and k_2 differ by only 3-fold. For refolding near 0.5 M Gdn·HCl fitting the data to two phases is justified by the fact that there are clearly two phases at both higher and lower final denaturant concentrations and because attempts to fit the data to a single component (in addition to k_3) show systematic deviations in plots of the residuals.

Preliminary attempts have been made to compare the total kinetic signal changes to those observed at equilibrium. These kinds of comparisons are complicated by baseline uncertainties in the equilibrium transitions and by the different spectral bandwidths and optical paths used in the stopped-flow and scanning spectrofluorometers. The three folding kinetic phases appear to account for all the equilibrium signal change for refolding ending in the native protein baseline region (0−0.2 M final Gdn·HCl). Comparisons at other denaturant concentrations and for unfolding are inconclusive.

The specific activities of FKBP and CyP, $(k_{cat}/K_m)_{pep}$, were measured toward the peptide substrate Suc-AAPF-pNA according to Harrison and Stein (1990), using the equation:

$$(k_{cat}/K_m)_{pep} = (k_{obs} - k_0)/[PPI] \quad (2)$$

where k_{obs} is the observed first-order rate constant measured by absorbance at 390 nm, k_0 is the uncatalyzed rate, and [PPI] is the concentration of prolyl isomerase (CyP or FKBP) used in the assay. The catalytic efficiency of folded FKBP or CyP toward a protein substrate (FKBP in the process of refolding) was measured in a similar manner for the slow (τ_1) and intermediate (τ_2) kinetic phases in FKBP folding using

$$(k_{cat}/K_m)_{prot,i} = (k_i - k_{i0})/[PPI] \quad (3)$$

where $(k_{cat}/K_m)_{prot,i}$ is the catalytic efficiency for catalysis of folding for kinetic phase *i*, k_i is the apparent rate for phase *i*, k_{i0} the apparent rate for phase *i* in the absence of added folded PPI, and [PPI] is the concentration of added folded prolyl isomerase (FKBP or CyP). Catalytic efficiencies ($k_{cat}/K_m)_{prot,i}$ toward particular kinetic phases in folding of protein substrates were determined from plots of k_i vs [PPI], where $(k_{cat}/K_m)_{prot,i}$ and k_{i0} are the slope and intercept of the plots, respectively.

A slowly drifting baseline limits the accuracy of measurements of FKBP folding rates in the presence of high concentrations of native FKBP. The cause of the drift is not known. Interestingly, the baseline drift is eliminated for FKBP refolding in the presence of both native FKBP and FK506. There is little or no baseline drift for native FKBP alone.

Refolding of FKBP in the presence of FK506, an inhibitor of the prolyl isomerase activity of FKBP, was carried out in the same manner as folding in the absence of FK506. A stock solution of FK506 dissolved in HPLC-grade methanol (Standaert et al., 1990) was diluted into the refolding buffer to give final concentrations of 45 μM FK506 and 1.9% v/v methanol. Controls with fully folded FKBP showed that binding of FK506 to FKBP was complete within the mixing dead time (5 s).

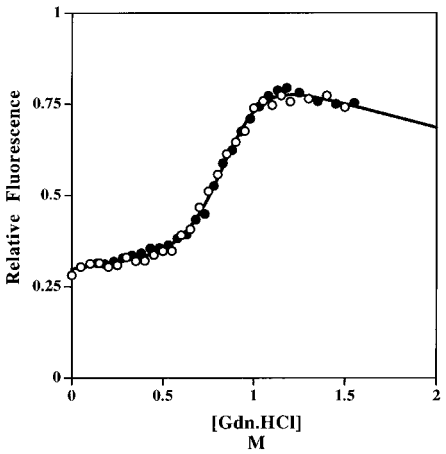

FIGURE 1: Guanidine hydrochloride-induced equilibrium unfolding transition for FKBP. Unfolding is monitored by fluorescence at 350 nm with excitation at 285 nm. Fluorescence intensities of the protein solutions are given relative to the fluorescence of an equimolar amount of NATA. Filled circles are measurements made by adding folded FKBP to buffer containing the indicated concentration of Gdn·HCl. Open circles are measurements obtained by diluting samples of Gdn·HCl-unfolded FKBP to the indicated Gdn·HCl concentration. The line passing through the data is a least squares fit of the data to a two-state linear extrapolation model of unfolding (Pace, 1986; Santoro & Bolen, 1988) with parameters of $\Delta G°_U(H_2O) = 4.6 \pm 0.3$ kcal mol^{-1} and $m \equiv d[\Delta G_U]/d[Gdn·HCl] = 5.4 \pm 0.4$ kcal mol^{-1} M^{-1}. Conditions are 0.02 M Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.0, 20 °C, and the indicated concentration of Gdn·HCl.

RESULTS

Equilibrium Thermodynamic Stability Measured by Gdn·HCl-Induced Unfolding. Figure 1 shows the fluorescence-detected Gdn·HCl-induced equilibrium unfolding transition for FKBP at 20 °C, pH 8.0. The transition is fully reversible since the measured fluorescence depends only on the final denaturant concentration and not on whether the sample was prepared from a stock solution of native protein or unfolded protein. Use of the linear extrapolation model to estimate the stability of the folded protein in the absence of denaturant gives an unfolding free energy of $\Delta G°_U(H_2O) = 4.6 \pm 0.3$ kcal mol^{-1}. Other parameters obtained from the linear extrapolation model are the transition midpoint, $C_m = 0.85 \pm 0.1$ M, and the denaturant dependence of the free energy, m ($\equiv d[\Delta G_U]/d[Gdn·HCl]$) $= 5.4 \pm 0.4$ kcal mol^{-1} M^{-1}. The value of m is much higher than expected for a globular protein of 12 kDa. Correlations between accessible surface areas and m values for 45 proteins suggest an m value near 3.0 for Gdn·HCl unfolding of a protein the size of FKBP (Myers et al., 1995). The high m value results in an unusually large free energy of unfolding for a protein with such a low transition midpoint. Specific activity has also been used to measure unfolding of FKBP (data not shown). Unfolding monitored by specific activity gives a transition with essentially the same shape as the fluorescence-detected transition shown in Figure 1. Changes in specific activity on unfolding and refolding are also fully reversible (Veeraraghavan, 1995).

Fluorescence-Detected Rates for Refolding and Unfolding of FKBP. By varying the final denaturant concentration, folding/unfolding kinetic experiments have been carried out

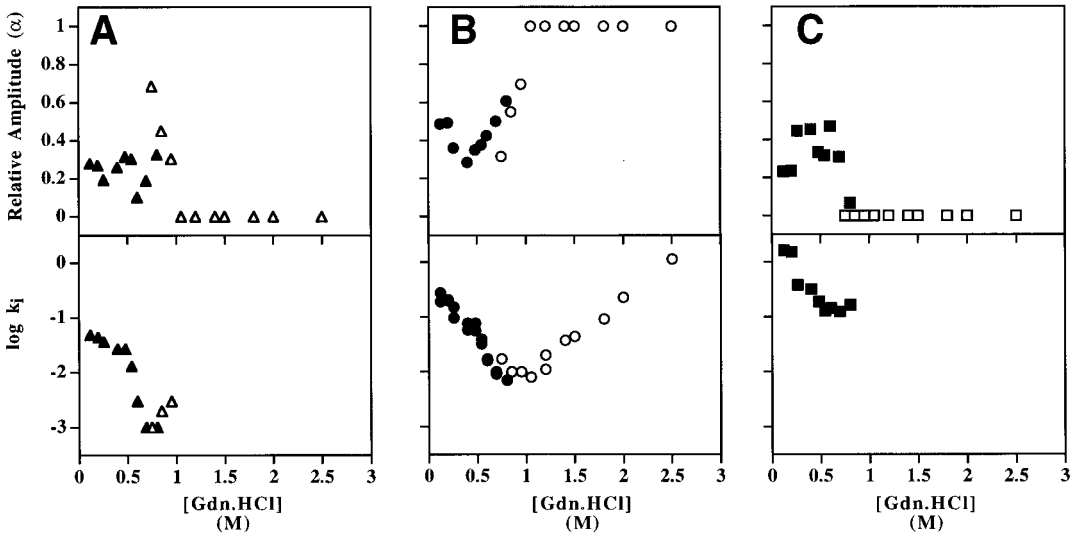

FIGURE 2: Guanidine hydrochloride dependence of the rates and amplitudes of the kinetic phases in folding/unfolding of FKBP: logarithm of the apparent rate constants ($k_i = 1/\tau_i$) for the folding and the unfolding phases (lower panels) and the associated amplitudes (upper panels) for kinetic phases (A) τ_1 (triangles), (B) τ_2 (circles), and (C) τ_3 (squares) as a function of final Gdn·HCl concentration. Closed symbols represent refolding data while open symbols are unfolding data. Amplitudes are given relative to the total fluorescence change detected in the kinetic experiments. Refolding was induced by dilution of a solution of 100 μM unfolded FKBP in 2 M Gdn·HCl with mixing buffer to give 6 μM FKBP and the indicated Gdn·HCl concentration. Unfolding was initiated by the dilution of 100 μM native FKBP into mixing buffer to give the indicated Gdn·HCl concentration and 6 μM FKBP. Mixing buffers were 0.02 M Tris, 1 mM EDTA, and 1 mM β-ME, pH 8.0, and contained sufficient Gdn·HCl to give the indicated final concentrations of Gdn·HCl. All data points are averages of three or more independent measurements. The errors in the rate constants, as estimated from the standard deviations, are typically 10% of the values reported.

for final refolding conditions that end below, within, and above the equilibrium unfolding transition zone. Three kinetic phases are detected. The guanidine hydrochloride concentration dependence of the apparent rate constants ($k_i = 1/\tau_i$) and relative amplitudes (α_i) for the kinetic phases is given in Figure 2. All three refolding phases have comparable amplitudes below 0.6 M Gdn·HCl, but above 0.8 M Gdn·HCl the amplitudes of the fast (τ_3) and slow (τ_1) phases decrease and that of the intermediate phase (τ_2) increases. Unfolding experiments ending near the transition midpoint occur with biphasic (τ_1, τ_2) kinetics (Figure 2A,B), but above the midpoint of the folding transition, a single unfolding phase (τ_2) is observed.

CyP Catalysis of FKBP Refolding. The rate for the slowest phase in refolding of FKBP (τ_1) is enhanced when the peptidyl−prolyl *cis*−*trans* isomerase, CyP, is included in the final refolding conditions. Thus, at least one factor governing the rate of phase τ_1 is proline *cis*−*trans* isomerization. The effect of adding increasing amounts of native CyP on the rate of the fluorescence-detected slow folding phase (τ_1) is shown in Figure 3A. The apparent rate constant ($k_1 = 1/\tau_1$) varies linearly with increasing CyP between 0 and 2 μM CyP. Between 2 and 8 μM CyP the apparent rate plateaus at a maximum value of about 2-fold the uncatalyzed rate. The catalytic efficiency of CyP toward phase τ_1 of FKBP refolding, estimated from a least squares fit of the data between 0 and 2 μM CyP, gives $k_{cat}/K_m = (2.8 \pm 0.2) \times 10^4$ M^{-1} s^{-1}. There was no significant change in the rate of the intermediate refolding phase (τ_2) in the presence of CyP (not shown).

FKBP Catalysis of FKBP Refolding. Since FKBP, like CyP, is a peptidyl−prolyl *cis*−*trans* isomerase, experiments were carried out to test whether FKBP catalyzed its own slow folding reactions. Addition of native FKBP to FKBP refolding reactions enhanced the rates of two of the three refolding phases. A modest enhancement of about 1.4-fold is observed for the rate of the intermediate (τ_2) refolding phase (data not shown). The apparent refolding rate for phase τ_1 increases with increasing concentrations of native FKBP between 0 and 4 μM FKBP and gives a rate enhancement of 1.8-fold at 4 μM FKBP (Figure 3B). Least squares fits to the data give estimates of $k_{cat}/K_m = (4.1 \pm 1) \times 10^4$ M^{-1} s^{-1} for the intermediate refolding phase (τ_2) (data not shown) and $k_{cat}/K_m = (6.0 \pm 0.2) \times 10^3$ M^{-1} s^{-1} for the slow phase (τ_1).

Autocatalysis of FKBP Folding. Since native FKBP catalyzes refolding of FKBP, catalytically active FKBP formed early in refolding may catalyze folding of more slowly refolding species. This possibility has been tested by measuring refolding rates for a series of experiments in which the final (refolding) concentration of FKBP was varied between 1 and 15 μM without the addition of native FKBP. For these experiments catalytically active FKBP must form by refolding of (initially) unfolded FKBP. As shown in Figure 4, the rate of the slow phase (τ_1) in folding increased with increasing FKBP concentration for experiments starting with all the FKBP unfolded. The results show that folding is autocatalytic: the products of folding, probably from phases τ_3 and τ_2, enhance the rate of the slow phase (τ_1). Autocatalysis of phase τ_1 in FKBP refolding is eliminated when FK506, an inhibitor of the FKBP prolyl isomerase activity, is included in the final refolding conditions (Figures 4 and 5).

DISCUSSION

Reversibility of Folding. In contrast to the irreversible effects of denaturants on CyP, unfolding of FKBP is fully reversible with respect to changes in the intrinsic fluorescence (Figure 1) and specific activity toward the tetrapeptide substrate, Suc-AAPF-pNA (data not shown). Reversibility is demonstrated by the fact that the relative fluorescence (or

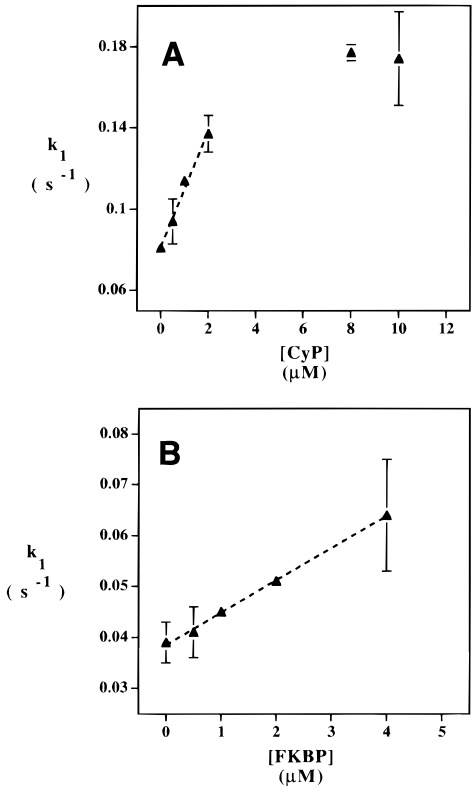

FIGURE 3: (A) Catalysis of the slow refolding phase (τ_1) of FKBP by cyclophilin (CyP). Increasing amounts of CyP were added to the refolding mixture, and folding was initiated by the dilution of 120 μM unfolded FKBP in 2 M Gdn·HCl. Fluorescence changes associated with the refolding reactions were monitored using stopped-flow fluorometry. The first-order rate constant, k_1 (=1/τ_1), is plotted against the final concentration of the added cyclophilin. The dashed line through the initial region of the plot (0−2 μM CyP) is a least squares fit to the equation $y = 0.082 + 0.028$ [CyP]. The slope of the line provides an estimate of the catalytic efficiency (k_{cat}/K_m) of CyP toward the slow refolding species. Final refolding conditions were as follows: 14.4 μM FKBP, 0.24 M Gdn·HCl, 0.02 M Tris, 1 mM EDTA, and 1 mM β-ME, pH 8.0, 20 °C. (B) Catalysis of FKBP folding by added native FKBP. The effect of adding increasing amounts of native FKBP on k_1 (=1/τ_1) for the slow refolding phase is plotted vs the concentration of added native FKBP. Refolding was initiated by dilution of a solution containing 175 μM unfolded FKBP in 2.0 M Gdn·HCl with buffer (0.02 M Tris.HCl, 1 mM EDTA, 1 mM β-ME, pH 8) to give 2 μM FKBP, and 0.24 M Gdn·HCl. The mixing buffer contained sufficient native FKBP to give the indicated native FKBP concentration in the final refolding conditions. The dashed line through the data (0−4 μM FKBP) is a least squares fit to the equation $y = 0.038 + 0.006$[FKBP]. The slope of the dashed lines provides an estimate of the catalytic efficiencies (k_{cat}/K_m) of FKBP toward the species which refold at a slow rate.

specific activity) depends only on the final concentration of denaturant and is independent of whether the sample was prepared from a stock solution of folded FKBP or unfolded FKBP. Because of the reversible unfolding transition, FKBP may be a more useful catalyst for some (*in vitro*) folding reactions than CyP despite the fact that k_{cat}/K_m of CyP is 2-fold to 3600-fold higher than that of FKBP toward proline-containing peptide substrates (Harrison & Stein, 1990). The utility of CyP as a catalyst of *in vitro* protein folding is limited by the time-dependent irreversible inactivation of CyP under the mildly denaturing conditions required for many folding intermediates to be prolyl isomerase substrates (Veeraraghavan & Nall, 1994; Veeraraghavan et al., 1995b). Under the same mildly denaturing conditions the specific

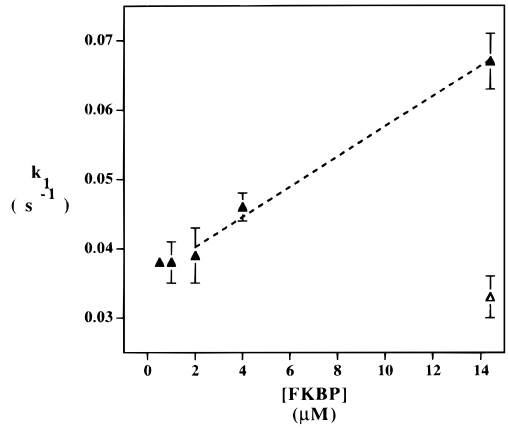

FIGURE 4: Autocatalysis of FKBP folding. The dependence of k_1 (=1/τ_1) for the slow refolding phase on the final FKBP concentration is given for experiments in which all the FKBP was (initially) unfolded (filled triangles). The initial and final refolding conditions were the same as in Figure 3 except that the amount of unfolded FKBP in the initial conditions was varied to give the final FKBP concentrations indicated on the *x*-axis after dilution of the denaturant. The open triangle at 14.4 μM FKBP is for refolding of FKBP in the presence of 45 μM FK506, an inhibitor of FKBP prolyl isomerase activity (see Figure 5). The dashed line through the filled triangles (2−14 μM FKBP) is a least squares fit to the equation $y = 0.036 + 0.002$[FKBP]. The slope of the dashed line provides an estimate of the catalytic efficiency (k_{cat}/K_m) of FKBP toward the slow refolding species.

activity of FKBP, while reduced, is time independent and FKBP activity is fully recovered on removing the denaturant.

Kinetic Phases and Mechanistic Aspects of Folding. All three kinetic phases in FKBP folding have rates which depend on denaturant concentration, suggesting coupling of the reactions responsible for the phases to denaturant-sensitive structure. The τ_2 phase, which is observed over the full range of folding and unfolding conditions, is probably closely associated with the major structure-forming reactions leading to thermodynamic stability. As expected for a kinetic process associated with equilibrium stability, the apparent rate constant ($k_2 = 1/\tau_2$) has a minimum value near the midpoint of the denaturant-induced equilibrium unfolding transition (Matthews, 1987; Matthews & Hurle, 1987). The apparent rate constants for phases τ_1 and τ_3 pass through minima at denaturant concentrations only slightly below the equilibrium transition midpoint, suggesting that the reactions that generate these phases may also be coupled to processes important to equilibrium stability.

FKBP Folding and Proline Isomerization. Since there are three kinetic phases for folding/unfolding of FKBP, a minimum of four species is involved. Species other than the native protein and the unfolded protein may be produced by kinetic traps such as proline isomerization which can lead to heterogeneity of the unfolded (or folded) forms. This is likely for FKBP which contains seven prolines out of a total of 107 amino acids (Standaert et al., 1990). Moreover, an unusual Pro−Pro sequence is found at positions 92 and 93. Isomerization of a Pro−Pro sequence in a protein not related to FKBP is known to generate very slow refolding forms of that protein (Grafl et al., 1986; Lang et al., 1986).

All seven of the proline residues in FKBP are in the *trans* configuration in the native state (Van Duyne et al., 1991, 1993). Isomerization of one or more of these prolines to non-native *cis* configurations in the unfolded protein could produce kinetic traps and generate slow refolding species.

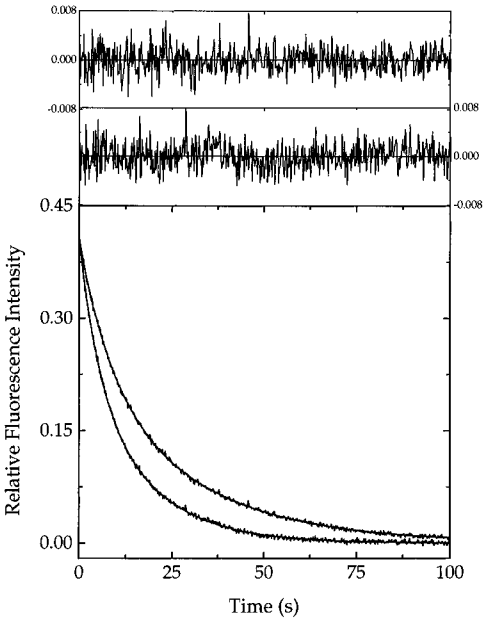

FIGURE 5: FK506 inhibition of autocatalysis of FKBP slow folding. FKBP unfolded in 2.2 M Gdn·HCl was refolded by mixing with buffer to give final refolding conditions of 0.24 M Gdn·HCl and 14.4 μM FKBP. The upper trace (bottom panel) is for refolding in the presence of 45 μM FK506, an inhibitor of the prolyl isomerase activity of FKBP. The lower trace (bottom panel) is for folding in the absence of the inhibitor. Corrected for an estimated 5 s mixing time, the least squares fit to the upper trace (bottom panel) gives $S(t) = 0.30\ \exp(-0.125t) + 0.25\ \exp(-0.037t)$. The fit to the lower trace gives $S(t) = 0.34\ \exp(-0.20t) + 0.21\ \exp(-0.063t)$. $S(t)$ is the fraction of the total kinetically detected signal change in the stopped-flow and manual mixing time range (4 ms to 6000 s), and t is the time in seconds. The fitting function contains two exponential terms, one for phase τ_1 and one for phase τ_2. The fast phase, τ_3, is readily detected by stopped-flow mixing but is complete within the dead time of the manual mixing experiments shown here (Figure 2). The uppermost panel gives a plot of the residuals of the fit for the upper trace (lower panel), and the middle panel gives a plot of the residuals of the fit for the lower trace (bottom panel). Other conditions are TEM buffer, pH 8, 20 °C.

The involvement of proline isomerization in phase τ_1 seems certain since the rate of this kinetic phase is enhanced in the presence of two distinct prolyl *cis*−*trans* isomerases, CyP and FKBP (Figure 3). In addition, the rate enhancement by FKBP is eliminated by FK506, an inhibitor of the FKBP prolyl isomerase activity (Figures 4 and 5). This shows that the FKBP prolyl isomerase activity is needed for catalysis of FKBP slow folding and rules out chaperonin-like effects or protein concentration as determinants of the slow folding rate. It is likely that proline isomerization is also a factor governing the rate of phase τ_2, since this phase is faster for folding in the presence of native FKBP (data not shown). Although phase τ_2 is not catalyzed by the other prolyl isomerase, CyP, there are at least two possible explanations: (1) the larger size of CyP compared with FKBP may block access of CyP to substrate prolines; 2) differences in catalysis by FKBP vs CyP may arise from differences in substrate specificity (Harrison & Stein, 1990).

Catalysis vs Autocatalysis of Folding. At first it seems surprising that the slow folding phase (τ_1) can be fit by an exponential decay function under conditions of significant autocatalysis (Figures 4 and 5) where the catalyst (FKBP) concentration changes during slow folding. Since the slow phase generates its own catalyst as a product, one might have expected more complex kinetic behavior. We believe the lack of complexity is because most of the FKBP activity is regained in the faster phases of folding (τ_2, τ_3), thus buffering FKBP activity over the course of phase τ_1. For example, if the fluorescence signals give a measure of the amount of activity regained, about 80% of the active FKBP is formed in the faster phases prior to phase τ_1 (Figure 2). As seen from Figure 3B, the remaining 20% change in concentration of FKBP formed in phase τ_1 will have a negligible effect on k_1 (i.e., from eq 3, $k_1 = k_{10} + [\text{FKBP}](k_{cat}/K_m)_{prot,1}$).

Despite the discovery of chaperonins and isomerases, the principle remains: the information specifying the three-dimensional structure of a protein is encoded by the amino acid sequence. As a class, chaperonins protect nascent polypeptides from improper (intermolecular) associations. This allows the incorrect associations to be avoided (or abbreviated) so that intramolecular folding proceeds. Thus, the chaperonins facilitate expression of sequence-encoded structural information by filtering out intermolecular interference. In contrast, isomerases act as filters of intramolecular interference. Intrinsically slow processes, in particular, proline isomerization, lead to formation of kinetically trapped, misfolded species. Isomerases catalyze the intrinsically slow processes so that the kinetically trapped species no longer form. Thus, both chaperonins and isomerases facilitate proper folding by discouraging competing off-pathway interactions. Although there are exceptions, chaperonins act primarily to prevent (incorrect) *inter*molecular associations, while isomerases prevent (incorrect) *intra*molecular associations. In both cases the formation of correct, sequence-encoded interactions is facilitated. Unlike the chaperonins, isomerases also catalyze on-pathway folding reactions, for example, when an obligatory slow step in folding involves proline isomerization.

Proteins can catalyze their own folding by either intramolecular or intermolecular mechanisms. There are now examples of each type. Proenzyme folding can be facilitated by the propeptide (Ikemura et al., 1987). For example, the propeptide inhibitor component of a propeptidase enhances the rate of folding of the mature protease peptide component, and following folding, the propeptide inhibitor is hydrolyzed in an intramolecular autocatalytic reaction generating a mature protease (Baker et al., 1992a,b; Bryan et al., 1992; Strausberg et al., 1993). Intramolecular catalysis occurs in folding of ribonuclease A where a slow isomerization reaction is catalyzed by a structure formed in a slow folding intermediate (Schmid, 1986). A reaction involving proline isomerization in folding of dihydrofolate reductase provides a second example of intramolecular catalysis of folding (Texter et al., 1992). The present results provide an example of intermolecular autocatalysis of a folding reaction.

CONCLUSIONS

Unfolding of FKBP is uncomplicated and occurs in a single kinetic phase for kinetic experiments ending above the midpoint of the equilibrium unfolding transition zone. Folding of FKBP occurs in three kinetic phases, a fast phase (τ_3) and two slower folding phases (τ_1 and τ_2). The addition of a native prolyl isomerase, CyP, to FKBP refolding reactions catalyzes the slowest refolding phase, τ_1. FKBP is itself a prolyl isomerase, which when added as folded native protein to FKBP refolding reactions catalyzes phases τ_1 and τ_2. Even in the absence of added native FKBP, the

slowest phase, τ_1, is catalyzed by FKBP formed in faster folding phases. The autocatalytic effect of FKBP on its own slow folding is eliminated for folding in the presence of FK506, an inhibitor of FKBP prolyl isomerase activity. Thus, the FKBP prolyl isomerase activity produced on folding of FKBP catalyzes formation of additional FKBP prolyl isomerase.

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