Mechanism of Assembly of a Leucine Zipper Domain

Hans Wendt, Antonio Baici, and Hans Rudolf Bosshard*

Department of Biochemistry, University of Zürich
CH-8057 Zürich, Switzerland

Received March 14, 1994

Leucine zippers are composed of amphipathic α-helices containing heptad repeats (*abcdefg*) in which hydrophobic residues are frequent at *a* and *d*. The motif is found in many DNA-binding proteins.[1] The variety of combinatorial interactions between different α-helices provides for the control of function of basic-region leucine zipper transcription factors.[2] Physical studies established that interhelical hydrophobic interactions between residues in the *a* and *d* positions as well as inter- and intrachain electrostatic interactions govern the formation and stability of homomeric and heteromeric leucine zippers.[2,3] The kinetics and mechanisms by which the separate chains of a leucine zipper assemble are not known. Here we show that the assembly of a dimeric leucine zipper involves conformational rearrangements after the initial association of chains.

Residues 249–281 of the yeast transcriptional activator protein GCN4, named peptide GCN4-p1,[4a] form a dimeric, parallel coiled coil.[4b,5] The helices are only stable when folded in the coiled coil conformation but not as individual monomeric peptide chains.[6] Therefore, it seems unlikely that the two unfolded chains (M) associate to the native leucine zipper dimer (D) in a single step. Rather, monomers (perhaps in a partially folded state) probably associate in a concentration-dependent reaction to a dimeric intermediate (D*), which then relaxes to the native dimer (D) via one or more monomolecular steps. A minimal model of the mechanism is

$$M + M \overset{\tau_1}{\rightleftarrows} D^* \overset{\tau_2}{\rightleftarrows} D \qquad (1)$$

The two reaction steps are characterized by the relaxation times τ_1 and τ_2. τ_1 depends on the initial peptide concentration, while τ_2 is apparently concentration-independent unless the two steps are strongly coupled ($\tau_1 \simeq \tau_2$).

We synthesized peptide FLU-GGG-GCN4-p1 corresponding to GCN4-p1 with a fluorescein group (FLU) attached to the N-terminus via a triglycine spacer (GGG).[7] Fluorescence emission of FLU-GGG-GCN4-p1 is quenched, presumably through self-quenching of the two FLU groups in the parallel coiled coil dimer, when compared to the unfolded peptide in 8 M urea or to a peptide that does not form a coiled coil (Figure 1, inset). Quenching is concentration dependent according to the monomer ⇄ dimer equilibrium: $M + M \rightleftarrows D$ ($K_d = M^2/D$). From the quench curve of Figure 1 we calculate $K_d = 0.48 \pm 0.23$ μM.[8] $K_d = 0.57 \pm 0.19$ μM was obtained independently from the change of ellipticity with concentration of GCN4-p1 observed by CD spectroscopy (not shown).[9] The good agreement of K_d values indicates that fluorescence quenching and ellipticity change describe the same equilibrium process and that the FLU-label does not interfere with leucine zipper formation.

The time course of association and dissociation was studied by following the relaxation kinetics after disturbance of a preexisting equilibrium. When FLU-GGG-GCN4-p1 was rapidly diluted, relaxation to the new equilibrium led to a time-dependent increase of fluorescence (Figure 2).[10] The fluorescence change could be described by two relaxation processes according to the equation $F = A_1[1 - (\exp(-t/\tau_1)] + A_2[1 - \exp(-t/\tau_2)] + A_0$. A_1 and A_2 are the amplitudes for the fast and the slow phase, respectively, and A_0 is the fluorescence at $t = 0$. τ_1 and τ_2 are functions of the individual rate constants which make up the overall equilibrium.[11] Because the two phases were only weakly coupled (τ_2/τ_1 ca. 5–7) and the amplitudes were of similar magnitude, a fit to a single exponential relaxation process could be excluded with certitude. However, unlike that predicted by mechanism 1, τ_1 did not depend on the initial peptide concentration within the range of 0.9–3.6 μM (Table 1). Mechanism 1 may be expanded by an additional concentration-independent step to account for this observation:

$$M + M \overset{\tau_0}{\rightleftarrows} D^{**} \overset{\tau_1}{\rightleftarrows} D^* \overset{\tau_2}{\rightleftarrows} D \qquad (2)$$

In mechanism 2, the initial concentration-dependent association reaction produces intermediate D** in which the two FLU groups are not close enough for self-quenching. Formation of D** (τ_0) could therefore not be seen as a concentration-dependent change of fluorescence. τ_1 and τ_2 pertain to the concentration-independent conformational rearrangements $D^{**} \rightleftarrows D^* \rightleftarrows D$.

We cannot definitely exclude the simpler mechanism 1 because the association rate constant may be too small to render τ_1 of mechanism 1 concentration-dependent under the conditions of the experiment. If in mechanism 1 $\tau_1 \ll \tau_2$, then $1/\tau_1 \simeq 4k_1M_\infty + k_{-1}$, where k_1 and k_{-1} are the association and dissociation rate constants and M_∞ is the concentration of monomeric peptide at

* Author for correspondence: phone, +41 1 257 5540; Fax, +41 1 363 79 47; e-mail, K297211@CZHRZU1A.bitnet.

(1) (a) Hope, I. A.; Struhl, K. *EMBO J.* **1987**, *6*, 2781–2784. (b) Landschulz, W. H.; Johnson, P. F.; McKnight, S. L. *Science* **1988**, *240*, 1759–1764.

(2) (a) Lamb, P.; McKnight, S. L. *Trends Biochem. Sci.* **1991**, *16*, 417–422. (b) Baxevanis, A. D.; Vinson, C. R. *Curr. Opin. Genet. Dev.* **1993**, *3*, 278–285.

(3) (a) Cohen, C.; Parry, D. A. D. *Proteins* **1990**, *7*, 1–15. (b) Hodges, R. S.; Semchuk, P. D.; Taneja, A. K.; Kay, C. M.; Parker, J. M. R.; Mant, C. T. *Peptide Res.* **1988**, *1*, 19–30.

(4) (a) O'Shea, E. K.; Rutkowski, R.; Kim, P. S. *Science* **1989**, *243*, 538–542. (b) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539–544.

(5) Saudek, V.; Pastore, A.; Castiglione Morelli, M. A.; Frank, R.; Gausepohl, H.; Gibson, T. *Protein Eng.* **1991**, *4*, 519–529.

(6) Thompson, K. S.; Vinson, C. R.; Freire, E. *Biochemistry* **1993**, *32*, 5491–5496.

(7) GCN4-p1 (Ac-RMKQLEDKVEELLSKNYHLENEVARLKKLV-GER-NH_2) was synthesized on an Applied Biosystems synthesizer Model 430A, using the N^{α}-9-fluorenylmethoxycarbonyl protection strategy and the Rapid Amide Resin from DuPont. FLU-GGG-GCN4-p1 was obtained by elongation with triglycine, followed by reaction with 5-(and 6-)carboxyfluorescein-*N*-succinimidyl ester (Molecular Probes, Eugene, OR). Peptides were purified by reversed-phase HPLC on a C8-column (0.1% trifluoroacetic acid/acetonitrile gradient) and analyzed by ion spray mass spectrometry and amino acid analysis.

(8) $F = \alpha M + \beta D$, where M and D are concentrations of monomer and dimer, respectively, α = molar fluorescence of monomer, and β = molar fluorescence of dimer. $\alpha = 11.73/\mu M$ was obtained from the fluorescence spectrum of FLU-GGG-EYEALEPKLAALEPKLQALEKKLEALEHG (Figure 1 inset), which has a random coil structure and cannot dimerize to a coiled coil (Leder, L.; Wendt, H.; Schwab, C.; Jelesarov, I.; Bornhauser, S.; Ackermann, F.; Bosshard, H. R. *Eur. J. Biochem.* **1994**, *219*, 73–81). With nominal peptide concentration $M_0 = M + 2D$ and $K_d = M^2/D$, F can be expressed by $4z^2 - z(4nM_0 + nK_d) + n^2M_0^2 = 0$, where $n = \beta - 2\alpha$ and $z = F - \alpha M_0$. This equation was fitted to measured F to obtain K_d and β by nonlinear regression (program SigmaPlot, Jandel Scientific).

(9) Ellipticities of GCN4-p1 were measured in a JASCO J-500 C spectropolarimeter. The equilibrium state is described by $(\theta - \theta_{min})/(\theta_{max} - \theta_{min}) = 2M_0B/(K_d + B)$. θ is the molar ellipticity at 220 nm, $B = 1 - (\theta - \theta_{min})/(\theta_{max} - \theta_{min})$, and $\theta_{min} = 2340$ deg^{-1} M^{-1} cm^{-1} (Cheng, Y.-H.; Yang, J. T.; Martinez, H. M. *Biochemistry* **1972**, *11*, 4120–4132). The equation was fitted to θ to obtain $K_d = 0.57 \pm 0.19$ μM and $\theta_{max} = 27\,200 \pm 800$ deg M^{-1} cm^{-1}.

(10) Experiments performed at pH 7.0 and 25 °C with a SF-61 stopped flow spectrofluorimeter (High Tech Scientific Ltd., Salisbury, U.K.); excitation at 492 nm, emission above 530 nm (cut-off filter OG530), dead time 1 ms. τ_1 (fast phase) and τ_2 (slow phase) were averaged from 6–10 single measurements obtained by 1:1 dilution of solutions of initial nominal peptide concentrations 3.6, 2.7, 1.8, and 0.9 μM. The reason for the narrow concentration range is that concentrations above 4 μM produced considerable inner filter effects, and the signal-to-noise ratio became very low below 0.5 μM. Experiments were repeated at ionic strengths (μ) of 176 (0.1 M sodium phosphate pH 7.0), 18, 118, and 518 mM (10 mM sodium phosphate pH 7.0, μ adjusted with NaCl).

(11) See: Bernasconi, C. F. *Relaxation Kinetics*; Academic Press: New York, 1961; pp 20–36 and 76–80. The error of $1/\tau$ was <6% in our experiments.

0002-7863/94/1516-6973$04.50/0

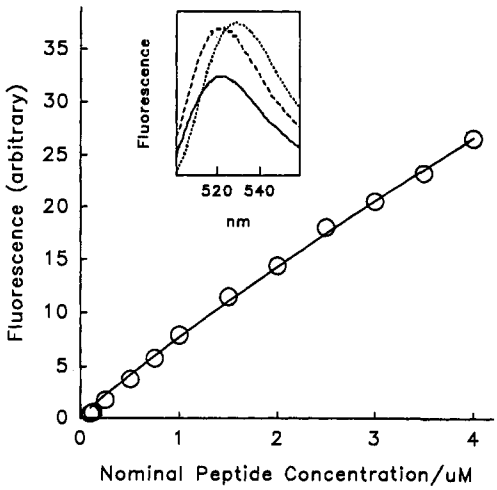

Figure 1. Concentration dependence of fluorescence emission at 522 nm (excitation 492 nm) of FLU-GGG-GCN4-p1 in 0.1 M sodium phosphate pH 7.0, 25 °C. Solid line calculated for $K_d = 0.48\ \mu M$, $\alpha = 11.73\ \mu M^{-1}$, $\beta = 10.53\ \mu M^{-1}$.[8] Inset: Fluorescence emission spectra of 1 μM solutions of FLU-GGG-GCN4-p1 (solid line) and a FLU-labeled peptide unable to form a coiled coil[8] (dashed line) in 0.1 M sodium phosphate pH 7.0 and of FLU-GGG-GCN4-p1 in 8 M urea (dotted line).

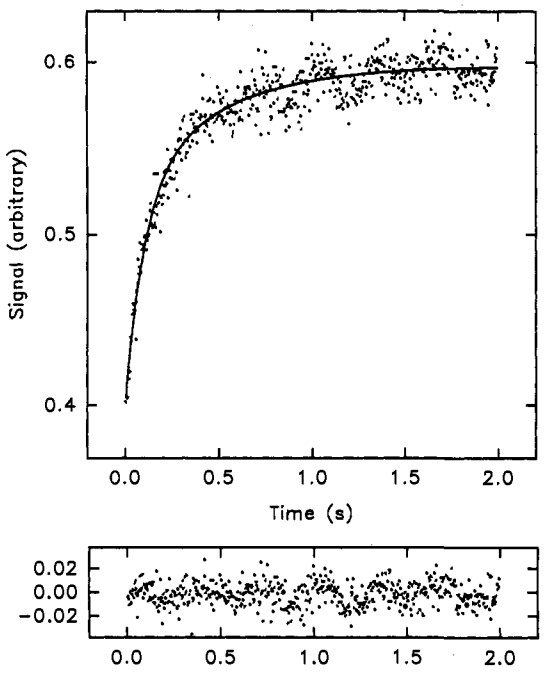

Figure 2. Relaxation to new equilibrium of 2.7 μM FLU-GGG-GCN4-p1 in 0.1 M sodium phosphate pH 7.0, 25 °C, $\mu = 176$ mM, after 2-fold dilution with same buffer. The signal change corresponds to a fluorescence increase.[10] Solid line calculated for $\tau_1 = 83$ ms, $\tau_2 = 410$ ms, $A_1 = 0.119$, $A_2 = 0.079$, $A_0 = 0.40$. Lower panel: residuals of calculated fit.

the new equilibrium.[12] This equation is an approximation for small displacements from equilibrium.[11] A plot of $1/\tau_1$ against M_∞ yields k_1 from the slope. In our experiments, the slope would have to be $>10^5\ M^{-1}\ s^{-1}$ to make $1/\tau_1$ appear concentration-dependent within the limit of the error of τ and the concentration range tested. Thus, mechanism 1 may apply if k_1 is considerably smaller than $10^5\ M^{-1}\ s^{-1}$. Such a small association rate constant would be untypical for the initial association step. For comparison, the bimolecular association rate constant for association of dimeric P22 Arc repressor from unfolded monomers is $\sim 10^7\ M^{-1}\ s^{-1}$.[13]

(12) $M_\infty = (-K_d + (K_d^2 + 8K_dM_0)^{1/2})/4$, M_0 = nominal concentration of peptide after dilution.

Table 1. Relaxation Times for Fast and Slow Phase

M_0[a] (μM)	μ[b] (μM)	τ_1 (ms)	τ_2 (ms)
3.6	176	105 ± 8	676 ± 183
2.7	176	83 ± 10	410 ± 34
1.8	176	112 ± 13	633 ± 120
0.9	176	84 ± 14	585 ± 34
c	18	71 ± 16	476 ± 159
c	118	68 ± 18	364 ± 53
c	518	71 ± 15	361 ± 39

[a] Nominal peptide concentration before rapid dilution. [b] Calculated assuming activity coefficients of 1. [c] Mean of values obtained at M_0 = 3.6, 2.7, and 1.8 μM.

Our major conclusion is that the assembly of the leucine zipper progresses through at least one kinetically discernible step of conformational reorganization. The reorganization must follow the initial association of chains because the helices are only stable in the coiled coil dimer conformation.[6] Computer simulation indicated that the folding of a leucine zipper may proceed through a continuum of conformational transitions.[14] A slow concentration-independent reorganization step was seen also in the only previous study of coiled coil formation in which refolding of α-tropomyosin from urea-denatured chains was followed by stopped-flow CD.[15] The kinetically defined intermediates D** and/or D* are probably ensembles of dimers in which the hydrophobic interface is not yet well developed, the chains are staggered, and the ends frayed. The heterogeneous nature of the intermediates is supported by a considerable variation of the amplitude ratio, $A_1/A_2 = 1.54 \pm 0.92$ (average for all experiments). Conformational rearrangements involve breaking of transient noncovalent interactions and formation of more stable inter- and intrachain bonds. This is thought to be a slower process than the initial association of chains.

Interhelical electrostatic interactions between residues in *e* and *g* positions are seen in the crystal of GCN4-p1.[4a] Because relaxation times do not depend on ionic strength (Table 1), electrostatic interactions do not contribute directly to the kinetic processes observed here. Other investigators have suggested that repulsion between equally charged residues rather than electrostatic attractions govern the stability of coiled coils.[16] To better understand the role of ionic bonds one needs to know the ionic strength dependence of K_d and of the rate constants of the initial association and dissociation reaction.

The approach presented opens the way to assess the influence of different heptad positions on the time course of assembly and to determine activation energies of the conformational rearrangement step(s). In experiments with model peptides we observed that residues in positions *a* and *d* contribute to very different degrees to variations in τ_1 and τ_2.[17] In the case of a model leucine zipper that forms a trimer in the crystal,[16c] relaxation measurements indicated dimerization followed by conformational reorganizations.[17] The kinetics of assembly of GCN4 with its DNA target may be studied with appropriately fluorescence-labeled basic-region leucine zipper peptides.[18]

(13) Milla, M. E.; Sauer, R. T. *Biochemistry* **1994**, *33*, 1125–1133.
(14) (a) Rey, A.; Skolnick, J. *J. Chem. Phys.* **1994**, *100*, 2267–2276. (b) Vieth, M.; Kolinski, A.; Brooks III, C. L.; Skolnick, J. *J. Mol. Biol.* **1994**, *237*, 361–367.
(15) Mo, J. M.; Holtzer, M. E.; Holtzer, A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 916–920.
(16) (a) O'Shea, E. K.; Rutkowski, R.; Kim, P. S. *Cell* **1992**, *68*, 699–708. (b) O'Shea, E. K.; Lumb, K. J.; Kim, P. S. *Curr. Biol.* **1993**, *3*, 658–667. (c) Lovejoy, B.; Choe, S.; Cascio, D., McRorie, D. K.; DeGrado, W. F.; Eisenberg, D. *Science* **1993**, *259*, 1288–1293.
(17) Manuscript in preparation.
(18) We thank Christine Berger for help with initial experiments and fruitful discussions. Work supported in part by the Swiss National Science Foundation (grant 31-36149.92).