Prolyl Isomerization as a Molecular Memory in the Allosteric Regulation of the Signal Adapter Protein c-CrkII

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Background: Signaling by chicken c-CrkII (cellular CT10 regulator of kinase) involves native state prolyl isomerization.

Results: Dynamic domain interactions are coupled with trans-to-cis isomerization at Pro-238 and down-regulation of chicken c-CrkII.

Conclusion: The linkage between protein folding, prolyl isomerization, and function of c-CrkII is explained by a kinetic six-species reaction mechanism.

Significance: The linkage with prolyl isomerization attenuates an allosteric transition and provides a molecular memory.

The signal adapter protein c-CrkII (cellular CT10 regulator of kinase) propagates signals from oncogenic tyrosine kinases to cellular targets (1–5). It consists of an SH2 domain followed by two SH3 domains. SH2 interacts with Tyr(P)-containing proteins, the N-terminal SH3 domain (SH3N) binds to proline-rich targets, and the C-terminal SH3 domain (SH3C) controls this binding activity (6–10). For chicken c-CrkII, two alternate forms have been detected by NMR spectroscopy (7, 9). In the open form, the substrate-binding site of the SH3C domain, the trans form is slightly favored, but in the presence of the interdomain linker and the SH3N domain (in SH3N-SH3C), the conformational equilibrium at Pro-238 is shifted toward cis. Surprisingly, the cis form was not detected in the NMR structure of human c-CrkII (6). This difference between human and chicken c-CrkII could be traced back to two differences in sequence: position 239 is occupied by Ile in the human and Phe in the chicken protein, and position 272 is occupied by Val and Met, respectively. The corresponding substitutions I239F and V272M in combination were necessary and sufficient to induce the low affinity form with a cis Pro-238 in human c-CrkII as well (11).

The transition between the two states is regulated by a native state prolyl isomerization at Pro-238, which is located at the N-terminal end of the SH3C domain, right after the interdomain linker. With cis Pro-238, c-CrkII adopts the closed, low affinity form, whereas with trans Pro-238, the open, high affinity form is populated. In the isolated SH3C domain, the trans form is slightly favored, but in the presence of the interdomain linker and the SH3N domain (in SH3N-SH3C), the conformational equilibrium at Pro-238 is shifted toward cis. Surprisingly, the cis form was not detected in the NMR structure of human c-CrkII (6). This difference between human and chicken c-CrkII could be traced back to two differences in sequence: position 239 is occupied by Ile in the human and Phe in the chicken protein, and position 272 is occupied by Val and Met, respectively. The corresponding substitutions I239F and V272M in combination were necessary and sufficient to induce the low affinity form with a cis Pro-238 in human c-CrkII as well (11).

In the low affinity form, interactions must exist between the SH3N and SH3C domains to shift the cis/trans equilibrium at Pro-238 toward cis and, concomitantly, to lower the substrate affinity of SH3N. Such interactions could, however, not be detected. In the SH3N-SH3C protein, the two domains showed the same equilibrium stability and the same folding kinetics as in isolation (11).

Here, we employed advanced kinetic techniques, in particular double-mixing experiments, to uncover domain interactions in c-CrkII and to elucidate how they are linked energetically with Pro-238 cis/trans isomerization in SH3C and with substrate binding to the SH3N domain. Based on these data and on kinetic simulations, we present a detailed mechanism that reveals the role of prolyl isomerization for the folding and the function of chicken c-CrkII, and we propose that the intrinsically slow isomerization at Pro-238 functions as a molecular memory, possibly as an attenuator, during the allosteric regulation of c-CrkII signaling.
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EXPERIMENTAL PROCEDURES

Materials—GdmCl ultra pure was purchased from MP Biomedicals (Santa Ana, CA). All other chemicals were purchased from Grüssing (Filsing, Germany).

Protein Expression and Purification—All constructs used in this study were cloned into pET11a as His6-Smt3-fusion proteins (SUMO protein from Saccharomyces cerevisiae). Site-directed mutagenesis was done by QuikChange PCR. Protein expression was performed in Escherichia coli BL21 (DE3) and protein purification as previously described (11). Essentially, cells were grown in double-yeast-tryptone medium at 37 °C. At an $A_{600} = 0.6$, protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (final concentration, 1 mM) for 3.5 h. Cells were harvested by centrifugation (GS-3 rotor, 5,000 rpm, 20 min), homogenized in lysis buffer (50 mM Tris/HCl, pH 8.0, 0.1 M NaCl, 10 mM EDTA) and disrupted using a microfluidizer. After centrifugation (SS-34 rotor, 15,000 rpm, 45 min), the supernatant containing the soluble fusion proteins was applied to a 5-ml Ni-NTA column (Qiagen), washed with 20 column volumes (50 mM Na$_2$HPO$_4$, 100 mM NaCl, 10 mM imidazole, pH 8.0) and eluted with 20 ml buffer (50 mM Na$_2$HPO$_4$, 100 mM NaCl, 250 mM imidazole, pH 8.0). The His$_6$-Smt3-tag was cleaved by His$_6$-Senp2 overnight while dialyzing against buffer without imidazole (50 mM Na$_2$HPO$_4$, 100 mM NaCl, pH 8.0, 0.5 mM DTT). A second Ni-NTA step in the absence of imidazole was employed to remove the His$_6$-Smt3 and His$_6$-Senp2. The tag-free protein in the flow through was concentrated and applied to a 16/600 Superdex 75 prep grade gel filtration column in 0.1 M potassium phosphate, pH 7.4.

The C3G-derived peptide of the sequence MYYDNSPPPALPPKKRQSAPS was also cloned into pET11 as a Smt3 fusion peptide and purified as described previously (11). The peptide was purified under denaturing conditions during the first Ni-NTA step (50 mM Na$_2$HPO$_4$, 100 mM NaCl, 6.0 M GdmCl, 10 mM imidazole, pH 8.0), and the His$_6$-Smt3-tag was cleaved after the gel filtration. The final purification step was the second Ni-NTA step.

Stopped Flow Kinetic Experiments—All the double-mixing experiments were performed using a SX17 MV sequential mixing, stopped flow fluorescence spectrophotometer (Applied Photophysics). The first mixing was performed with an 11-fold dilution, and the second was performed with a 6-fold dilution. The GdmCl concentrations of stock solutions were determined by measuring the refractive index (12), and those after the first and second mixing step were determined by using the dilution factors of the stopped flow apparatus. All kinetics were measured at least eight times under identical conditions, averaged, and fitted to exponential functions to obtain the amplitudes and the rate constants of the monitored reaction. These amplitudes were plotted as a function of the time lapse between the first and second mixing, and the traces again were analyzed using exponential functions. This gives information about the progress of the reaction that was initiated by the first mixing. The dead time of the instrumental setup was determined to be 80 ms in the double-mixing mode.

Interrupted Refolding—To analyze the refolding reaction, the proteins (33 μM solutions) were first denatured completely in 0.1 M potassium phosphate, 5.0 M GdmCl, pH 7.4, at 15 °C for at least 1 h. Refolding was initiated by 11-fold dilution with 0.1 M potassium phosphate, pH 7.4, at 15 °C. After different times, samples were mixed again with unfolding buffer to reach final conditions of 0.1 M potassium phosphate, 3.0 M GdmCl, pH 7.4, at 15 °C. The fluorescence change during this unfolding reaction of 0.5 μM protein was monitored above 320 nm after excitation at 280 nm.

Function-related Refolding Assay—To detect the folding state of SH3N after different times of refolding, the proteins (33 μM solutions) were denatured in 0.1 M potassium phosphate, 5.0 M GdmCl, pH 7.4, at 15 °C for at least 1 h. 11-fold dilution with 0.1 M potassium phosphate, pH 7.4, at 15 °C initiated refolding for defined time intervals, after which the protein was mixed with the C3G peptide (2.4 μM in the syringe) to monitor the association reaction of SH3N (in different protein variants) with the peptide. The final conditions were 0.5 μM protein, 2.0 μM C3G peptide in 0.1 M potassium phosphate, 0.08 M GdmCl, pH 7.4, at 15 °C. To monitor the binding reaction selectively, fluorescence was measured above 320 nm after excitation at 295 nm.

Interrupted Unfolding—The unfolding reactions of the proteins were analyzed starting from native protein (33 μM protein in 0.1 M potassium phosphate, pH 7.4, at 15 °C). 11-fold dilution with unfolding buffer initiated the unfolding reaction in 0.1 M potassium phosphate, 3.0 M GdmCl, pH 7.4, at 15 °C. After different times of unfolding, samples were transferred back to folding conditions by a second mixing step with buffer without GdmCl (0.1 M potassium phosphate, pH 7.4, at 15 °C). The final conditions were 0.5 μM protein in 0.1 M potassium phosphate, 0.5 M GdmCl, pH 7.4, at 15 °C.

To accelerate the unfolding reaction, in another set of experiments the native protein (33 μM in 0.1 M potassium phosphate, pH 7.4, at 15 °C) was mixed with 0.1 M glycine, 3.3 M GdmCl, pH 1.8 (to reach final conditions of 3.0 M GdmCl, pH 2.0). After different times of unfolding samples were transferred back to folding conditions by a second mixing step with buffer without GdmCl (0.1 M potassium phosphate, pH 7.4, at 15 °C). The final conditions were 0.5 μM protein in 0.1 M potassium phosphate, 0.5 M GdmCl, pH 7.4, at 15 °C by detecting the fluorescence above 320 nm after excitation at 280 nm. The same experiments were performed after 33 μM of the protein variants was incubated with 66 μM of the C3G peptide for at least 1 h at 15 °C.

Proline-limited Binding Reaction—The proline-limited binding reaction of chicken SH3N-SH3C and the P238A variant were monitored after manual mixing with a Jasco FP6500 fluorescence spectrophotometer. 1 μM of the SH3N-SH3C variant was incubated in 0.1 M potassium phosphate, pH 7.4, in the cuvette at 15 °C. Under constant stirring, increasing concentrations of the C3G peptide were added, and the fluorescence signal at 336 nm was monitored after excitation at 295 nm for 800 s. All measurements were repeated at least three times, averaged, and fitted to exponential functions to obtain the amplitude and the rate constant of the reaction.

Kinetic Simulations—Theoretical calculations of the folding, the ligand binding and the ligand binding as a function of the refolding time were performed using Scientist 2.01 (Micro-
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Math, Inc.). According to the six-species double-box model (see Fig. 8), the differential equations for all species were set up. Calculations were then performed for the same conditions as the corresponding experiments.

RESULTS

Domain Assembly Is Not Detected by Unfolding Assays—The conformational refolding reactions of the two SH3 domains of c-CrkII differ ~10-fold in rate. At low denaturant concentrations (0.5 M GdmCl) SH3N refolds in a single reaction with a rate of 0.8 s⁻¹. SH3C refolds more rapidly in two reactions with rates of 8 and 2 s⁻¹, which reflect the folding of the forms with trans and cis Pro-238, respectively (Fig. 1a). Both domains also show slow, denaturant-independent reactions in the 100-s time range that originate from the isomerization of prolines (11). SH3C and SH3N also differ strongly in the rate of unfolding, and at 3.1 M GdmCl, SH3C unfolds 100-fold faster than SH3N (Fig. 1b). SH3C thus shows much higher overall folding dynamics than SH3N.

These fluorescence-detected folding experiments did not provide evidence for a domain docking reaction, possibly because domain assembly is faster than domain folding or because it is not accompanied by a change in fluorescence. Late, spectroscopically silent processes in a folding reaction can be detected by a kinetic two-step assay that monitors the formation of fully folded molecules. This assay is based on the observation that protein molecules that have passed beyond the highest activation barrier during their assembly and thus have reached the fully folded state are separated from the unfolded state by this energy barrier. Thus, they unfold slowly when transferred to unfolding conditions. In the corresponding two-step assay for fully folded molecules (13), the unfolded protein is first mixed with refolding buffer to initiate its refolding. After variable times of refolding, the conditions are changed to unfolding, and the kinetics of the subsequent unfolding reactions are monitored. The amplitude of unfolding in this assay provides a direct measure for the fraction of molecules that had reached the native state at the time when refolding was stopped.

For the isolated SH3C domain of chicken c-CrkII, the formation of native molecules during refolding at 0.5 M GdmCl was probed by unfolding assays performed at 3.5 M GdmCl (Fig. 2a) in stopped flow double-mixing experiments. Under the assay conditions, native SH3C unfolds with a rate of 4 s⁻¹ (11). This rate is also observed in the unfolding assays at all times (Fig. 2a, upper panel), indicating that indeed the formation of fully folded molecules is monitored. Native SH3C forms in two reactions (Fig. 2a, lower panel) with rate constants of 9 and 0.026 s⁻¹ (supplemental Table S1), similar to the rates obtained from the fluorescence detected refolding kinetics. The experimental data in Fig. 2a deviate slightly from the biexponential fit in the range between 0.5 and 3 s. This probably reflects the refolding reaction of the cis Pro-238 species. Its amplitude is small, because the trans form is favored in the denatured protein, which is the initial state in these experiments (11). The P238A variant showed similar kinetics of formation of the native protein. They followed the biexponential fit more closely, because the cis Pro-238-dependent second conformational refolding reaction is missing (Fig. 2a). Again, the formation of native molecules parallels the fluorescence-detected folding kinetics. Similar results were obtained for the human SH3C domain (Fig. 2c). Together, the results in Fig. 2 (a and c) indicate that for the isolated SH3C domains of human and chicken c-CrkII, the fluorescence-detected kinetics represent the formation of the fully folded state and that there are no additional spectroscopically silent folding reactions.

The same stopped flow double-mixing analysis was performed for the chicken and the human SH3N-SH3C two-domain proteins (Fig. 2, b and d). Because SH3N unfolds 100-fold more slowly than SH3C, it virtually did not change its folding state within the 2-s time span that was used to follow the unfolding of SH3C. The unfolding assays thus monitored selectively the formation of the folded SH3C domain during the refolding of the SH3N-SH3C two-domain protein. The fully folded SH3C domains that were formed after various times of refolding all unfolded with the same rate constant of 4 s⁻¹ as the isolated SH3C domain (Fig. 2). As for the isolated SH3C domains, two major refolding reactions were observed for SH3C in the SH3N-SH3C two-domain protein, and the time course of the formation of the native SH3C domain paralleled
the fluorescence-detected folding of the isolated SH3C domain. Evidence for a domain assembly reaction with distinct kinetics could not be obtained. This shows that, in the folded SH3N-SH3C proteins, SH3C remains kinetically uncoupled from SH3N. The proline-limited, slow part of refolding is not abolished by the P238A substitution, but its time course is affected. This indicates that several prolines, including Pro-238, influence this reaction.

The rate of unfolding of the SH3C domain remained constant over the entire time course of refolding (Fig. 2) of the isolated SH3C domains, of the two-domain proteins, and of the P238A variants of both the chicken and the human protein. This provides strong evidence that domain folding is not followed by a tight domain docking reaction.

A Binding Assay Reveals an Impact of Pro-238 in SH3C on the Function of SH3N—The model for the functional interplay between the two SH3 domains of chicken c-CrkII assumes that the accessibility of the SH3N domain for substrates is decreased by a domain closing reaction linked with \( \text{trans} \rightarrow \text{cis} \) isomerization at Pro-238 in the SH3C domain. Here we used a peptide derived from the guanine nucleotide exchange factor C3G (Crk SH3-binding guanine-nucleotide releasing factor) (14–17) to monitor how the substrate affinity of SH3N develops during the refolding of the SH3N-SH3C two-domain protein.

Again, in a double mixing experiment, unfolded protein (isolated SH3N or SH3N-SH3C) was exposed to refolding pulses of increasing length and then, in the second step, was not unfolded as before but mixed with the C3G peptide to follow the formation of binding active molecules. The binding reaction itself is more than 100-fold faster than the folding of SH3N. The dissociation constant \( K_D \) of the complex between the isolated SH3N domain and the C3G peptide is 0.5 \( \mu \text{M} \), and in the presence of 2 \( \mu \text{M} \) peptide, complex formation is thus \( \sim75\% \) complete. The amplitude of the binding reaction therefore provides a measure for the formation of the native, binding-competent conformation during the initial folding pulse.
This function-related refolding assay was first performed with the isolated SH3N domains from human and chicken c-CrkII (Fig. 3, a and c). Most of them regain the functional native state rapidly, within a few seconds. Then a minor phase follows, which reflects the slow refolding of molecules with wrong proline isomers in the SH3N domain (supplemental Table S1). The rate constant of association with the C3G peptide remains virtually constant over the whole time range (Fig. 3, a and c, upper panels).

The peptide binding assay was then used to follow the folding of the two-domain protein SH3N-SH3C (Fig. 3, b and d). For human SH3N-SH3C, the amplitudes followed virtually the same time course as for the isolated SH3N domain (Fig. 3, c and d), evidently because SH3N folds in the two-domain protein as fast as in isolation and because the human two-domain protein remains in the domain-open, high affinity state after folding.

The chicken SH3N-SH3C protein shows a different profile (Fig. 3b). As for the human protein, a fast initial increase in the extent of peptide binding was observed, which reflects the conformational folding reaction of the SH3N domain. After refolding pulses longer than 10 s, the amplitude of binding did not increase further (as for isolated SH3N and for human SH3N-SH3C) but instead decreased in a slow reaction. This indicates that a process occurs after domain folding that obstructs the binding site and counteracts the formation of additional binding active molecules in the course of the slow proline-limited reaction. Because this effect on the SH3N domain is abolished by the P238A substitution (Fig. 3b), we conclude that it originates from a slow domain closure reaction, caused by Pro-238 trans \rightarrow cis isomerization.

In 90% of the unfolded molecules, the Gly-237–Pro-238 bond is expected to be in the trans conformation (18, 19). The rapid conformational folding thus leads to folded molecules that mostly adopt the high affinity, open state with trans Pro-238. In human SH3N-SH3C, the trans form of Pro-238 is favored in the folded state as well, and therefore the peptide binding site on SH3N remains continuously accessible.

**FIGURE 3.** Refolding of SH3C and SH3N-SH3C measured by binding assays. The apparent rates λ (upper panels) and amplitudes (lower panels) of C3G peptide binding are shown as a function of the time of refolding for chicken SH3N (a), chicken SH3N-SH3C (b), human SH3N (c), and human SH3N-SH3C (d). In b, data for the chicken SH3N-SH3C P238A variant are shown as open circles. Unfolded human SH3N (in 3.0 M GdmCl), chicken SH3N (in 5.4 M GdmCl), or human or chicken SH3N-SH3C (in 3.8 M GdmCl) were refolded by 11-fold dilution. After the indicated time intervals of refolding, the proteins were mixed with 2.4 μM C3G peptide in a further 6-fold dilution. The fluorescence increase upon binding was monitored above 320 nm, after excitation at 295 nm. Final conditions were 0.5 μM protein, 2 μM C3G peptide in 100 mM potassium phosphate, 0.05–0.08 M GdmCl, pH 7.4, at 15 °C. For each refolding interval, at least eight measurements were performed under identical conditions. The binding amplitudes were derived from fitting monoeponential functions to the binding kinetics. The parameters obtained from fitting biexponential functions to the amplitude profiles are shown in supplemental Table S1.
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In the folded chicken SH3N-SH3C protein, the cis form of Pro-238 is favored (7, 9, 11). Therefore, trans → cis isomerization at Pro-238 occurs after conformational refolding. It leads to the closed low affinity state and thus to the observed decrease in C3G peptide binding (Fig. 3b). The analysis by NMR spectroscopy of chicken c-CrkII variants suggested that Pro-238 is cis in ~90% of all molecules (9). If they were completely deficient in binding, the amplitudes of binding in Fig. 3b would decrease to a value close to zero. This is not the case, which demonstrates that the cis Pro-238 form interacts with the peptide as well, but with a significantly reduced affinity, as recently suggested (11).

In the folded form of chicken c-CrkII, most molecules are in the low affinity form with cis Pro-238. However, the C3G peptide binds preferentially to the trans Pro-238 form, and therefore incubation of native chicken SH3N-SH3C with the C3G peptide is expected to shift the equilibrium at Pro-238 toward the high affinity trans Pro-238 state. When the native protein is mixed with increasing concentrations of the C3G peptide, an additional slow binding reaction is in fact observed (Fig. 4). It reflects this Pro-238-limited shift in the cis/trans equilibrium, and its amplitude increases with the concentration of the C3G peptide up to 3 M (Fig. 5a). Above 4 M, it decreases again, because the molecules with cis Pro-238 associate with the C3G peptide in a fast reaction as well, but with a reduced affinity. Rapid direct binding to the low affinity cis state predominates at high peptide concentration, and coupling with Pro-238 cis → trans isomerization becomes less important. Human SH3N-SH3C (11) and the P238A variant of the chicken protein (Fig. 4) do not show such a proline-limited association reaction.

SH3N and SH3C Influence Each Other in Folded Chicken SH3N-SH3C—The function-related folding studies in Fig. 3b revealed a slow process after conformational domain folding that interferes with peptide binding to the SH3N domain and is controlled by Pro-238 trans → cis isomerization. To characterize the domain-assembled form of chicken c-CrkII further, we performed another set of double-mixing experiments, in this case starting with the native protein, which is mostly in the low affinity state with cis Pro-238 and contains all potential interactions between the two SH3 domains. In the first step, the protein was exposed to unfolding pulses (3.0 M GdmCl) of increasing length, and in the second step, this solution was transferred back to folding conditions (≤0.5 M GdmCl). The resulting fast conformational refolding reaction was monitored by fluorescence, and its amplitude was followed as a function of the time of unfolding in the first step. This reveals the properties of the assembly reaction between the folded domains.

The procedure was first employed to follow the unfolding of human SH3N-SH3C and of its isolated domains SH3N and SH3C (Fig. 5, a–c). Here, evidence for domain interactions in the initial folded state could not be obtained.

In the case of chicken c-CrkII, the isolated SH3C domain consists of a mixture of molecules with cis and trans Pro-238 isomers. They unfold with identical rates (Figs. 1 and 5e), but the trans Pro-238 species refolds faster than the cis Pro-238 species, and therefore two refolding reactions (with rate constants of 14 and 4.5 s⁻¹) were observed in the refolding assays (Fig. 5e). The trans form is strongly favored in the unfolded state, and therefore unfolding is followed by a slow shift of the cis/trans equilibrium toward trans, and the molecules with a cis Pro-238 decrease after extended times of unfolding.

As a part of the SH3N-SH3C protein, the chicken SH3C domain refolded in a single reaction (Fig. 5f). Possibly, this is caused by the presence of SH3N, which unfolds slowly and therefore remains largely native-like folded when the unfolding pulses are shorter than 5 s (Fig. 5d). The SH3C domain thus unfolds and refolds in the presence of a native SH3N domain. This probably accelerates the refolding of SH3C when Pro-238 is cis, because the cis form can be stabilized by the interactions with the folded SH3N domain. As a consequence, the refolding kinetics of the SH3C domains with cis or trans Pro-238 become more similar and can no longer be resolved experimentally. The time courses observed for the P238A variant (Fig. 5j) were similar to those of the wild-type protein.

How the refolding reaction of SH3C is affected by the folding state of SH3N or alternatively by the isomeric state of the prolines cannot be discriminated because these processes occur with similar rates at pH 7.4. Under acidic conditions, however, the conformational unfolding reactions of both SH3N and SH3C are strongly accelerated and thus kinetically separated from the prolyl isomerizations in the unfolded state, which are pH-independent.

At pH 2.0 and 3.0 M GdmCl, SH3C becomes unfolded within the dead time of the experiment (80 ms) (Fig. 6b), and SH3N unfolds within 1.5 s (Fig. 6a). After 150 ms, SH3C is thus unfolded, and SH3N is still folded. In the presence of the folded SH3N domain, the refolding amplitude of SH3C in the assay was increased by 60% (to 1.5 V; Fig. 5c). It decreased to 0.94 V in a reaction that showed the same kinetics as the unfolding of the SH3N domain. For the P238A variant of the chicken protein (Fig. 5c) and for the human protein (Fig. 5d), refolding of SH3C...
was independent of the folding state of the SH3N domain. The refolding of chicken SH3N-SH3C to the domain-closed form is thus accompanied by a stronger change in fluorescence than refolding to the domain-open state. This shows that domain closure in the chicken c-CrkII molecules with a cis Pro-238 leads to an increase in protein fluorescence.

Ligand Binding Shifts the cis/trans Equilibrium in Chicken c-CrkII—Most (90%) of the folded chicken SH3N-SH3C molecules exist in the closed form with cis Pro-238. Ligands such as the C3G peptide bind preferentially to the open trans form, and therefore ligand binding is expected to shift the cis/trans equilibrium at Pro-238 toward the trans state. This shift can now be visualized by the refolding assays after interrupted unfolding at pH 2.0, because molecules with a trans Pro-238 refold to the domain-open state with a smaller change in fluorescence.

To shift the cis/trans equilibrium at Pro-238 toward the binding-active trans form, we preincubated 33 μM chicken SH3N-SH3C with 66 μM C3G peptide for 60 min and then performed a double-mixing experiment as before (unfolding at pH 2.0, 3.0 M GdmCl, followed by refolding at pH 7.4, 0.5 M GdmCl). In fact, the initial amplitude of refolding was lowered from 160% as observed in the absence of the C3G peptide to 127% after preincubation with the C3G peptide (Fig. 7). This difference vanished after more than 20 s unfolding when the SH3N domain was unfolded as well. Obviously, incubation with the C3G peptide shifted the cis/trans equilibrium at Pro-238 toward the domain-open trans form, and in this form, the SH3C domain could not reassemble with the SH3N domain in the second step of the unfolding-refolding experiment. Incubation of the P238A variant with the C3G peptide did not affect its refolding amplitude, because this variant exists permanently in the domain-open form. Thus, there is a clear difference between human and chicken c-CrkII. In the human protein, the two SH3 domains are independent of each other, but in the chicken protein they engage in an interaction, which is established only when both domains

![Graph](https://example.com/graph.png)
are folded and when Pro-238 is in the cis conformation. It vanishes when Pro-238 is substituted by Ala. The domain interaction is accompanied by an increase in protein fluorescence and by an increase in stability, probably by accelerating the refolding of the cis Pro-238 species in the presence of folded SH3N. This shifts the cis/trans equilibrium at Pro-238 toward the cis form.

The domain interaction is highly dynamic, and its kinetics could not be resolved in conventional folding studies (11). It becomes apparent only when linked with a slow reaction, such as the folding of the SH3N domain or the isomerization of Pro-238.

A Six-species Scheme Models the Linkage between Domain Folding, Pro-238 Isomerization, and Ligand Binding—In chicken c-CrkII, protein folding, native state prolyl isomerization, and ligand binding are linked energetically. During folding, the cis/trans ratio at Pro-238 in the SH3C domain changes from 10/90 in the unfolded form to ~90/10 in the folded form (7, 9, 11, 18), and the Gibbs free energy required for this shift in equilibrium is provided by the conformational folding energy. The C3G peptide binds preferentially to the trans Pro-238 form and thus reverts this shift in the cis/trans equilibrium, presumably by interfering with interactions that stabilize the cis form.

The folding of c-CrkII from chicken can be modeled by a four-species box model (Fig. 8a), in which the two unfolded forms with Pro-238 in cis or in trans (U cis and U trans) coexist in a slow equilibrium. Both bind to the ligand, and the corresponding protein-ligand complexes (N cis-L and N trans-L) are in a slow, Pro-238-limited equilibrium as well. This leads to the six-species double-box model shown in Fig. 8.

The kinetic scheme in Fig. 8a is characterized by 14 microscopic rate constants, which are accessible from experiments. The ligand binds to the SH3N domain, and therefore its folding kinetics determine the left side of the kinetic scheme. SH3C does not contribute to the rate-limiting step of folding. The folding of SH3N is independent of Pro-238, and therefore k₃ =
The rate constants $k_{11}$ and $k_{12}$ (Fig. 8) for the association of SH3N with the ligand in the open $N_{\text{trans}}$ form are equal to those measured for the isolated SH3N domain (11). Folded SH3N-SH3C consists of $\sim$90% $N_{\text{cis}}$ and 10% $N_{\text{trans}}$, and we used the rates determined for its ligand association and dissociation (11) as the values for $k_9$ and $k_{10}$ (Fig. 8).

The microscopic rate constants for prolyl isomerization in the unfolded state, the native protein, and the protein-ligand complex (the vertical reactions in Fig. 8a) can also be derived from experimental data. For two-state reactions such as prolyl $cis \rightleftharpoons trans$ equilibriations, the equilibrium constant $K_{eq}$ and the apparent rate constant $\lambda$ must be known to calculate the two microscopic rate constants ($k_{\text{trans} \rightarrow cis}$ and $k_{cis \rightarrow trans}$) from a combination of Equations 1 and 2.

$$K_{eq} = \frac{[cis]}{[trans]} = \frac{k_{\text{trans} \rightarrow cis}}{k_{cis \rightarrow trans}} \quad \text{(Eq. 1)}$$

$$\lambda = k_{\text{trans} \rightarrow cis} + k_{cis \rightarrow trans} \quad \text{(Eq. 2)}$$

$U_{cis}$ and $U_{trans}$ refold with different rates, and the ratio of the relative amplitudes of their refolding reactions gives $K_{eq} = [U_{cis}]/[U_{trans}]$ as 11/89 (11), as in short peptides (19). The loss of the amplitude of refolding of the cis form with increasing time of unfolding (Fig. 5e, triangles) follows the rate of $cis \rightleftharpoons trans$ re-equilibration in the unfolded protein and gives a $\lambda$ value of 0.035 s$^{-1}$. By use of Equations 1 and 2, the microscopic rates $k_1$ and $k_2$ for $cis/trans$ interconversion in the unfolded state (Fig. 8b) were calculated.

In the folded protein, the $K_{eq}$ for $cis/trans$ equilibration $[N_{cis}]/[N_{trans}]$ is derived from the amplitude of ligand binding as a function of the refolding time (Fig. 3b). The P238A variant is permanently in the domain-open $N_{trans}$ state. The binding amplitude of 0.80 ± 0.04 V, reached after its folding, thus reflects its ligand binding equilibrium at the final concentrations of 0.5 mM $N_{trans}$ and 2 mM C3G peptide. The $K_D$ is 0.5 mM under the conditions of the experiment, and therefore, the observed amplitude results from 76% complex formation. The wild-type form of SH3N-SH3C reaches a final amplitude of ligand binding of 0.41 ± 0.03 V, equivalent to 39% complex formation. The observed amplitude reflects the sum of the complex formation by $N_{cis}$ and $N_{trans}$ which interact with the peptide with $K_D$ values of 3.6 and 0.5 mM, leading to 33 and 76% complex formation, respectively. The fractions of $[N_{cis}]$ and $[N_{trans}]$ are accordingly calculated by Equation 3.

$$0.33 \cdot [N_{cis}] + 0.76 \cdot (1 - [N_{cis}]) = 0.39 \quad \text{(Eq. 3)}$$

This leads to a $cis$ content in native SH3N-SH3C of 86% ± 5% (similar to the value estimated from the NMR analysis (9) and
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**FIGURE 9. Calculated time courses and comparison with experiments.** 
(a) calculated time courses during refolding for the species $U_{\text{trans}}$ (blue dashed line), $U_{\text{cis}}$ (red dashed line), $N_{\text{trans}}$ (blue continuous line), and $N_{\text{cis}}$ (red continuous line). The dashed black line represents the sum of all species. 
(b) calculated profile for the complex between SH3N-SH3C and C3G peptide (line, left ordinate), compared with the experimental data, taken from Fig. 3b (circles, right ordinate). 
(c) calculated amplitude profile for the slow, proline-limited binding reaction as a function of the peptide concentration (left ordinate, red dashed line) and the rate constants in Fig. 8 (right ordinate). 
(d) amplitude profile for the same reaction, calculated assuming a 4-fold reduced affinity of $N_{\text{cis}}$ for the ligand (dashed curve). For this calculation, the rate constant $k_1$ was 4-fold reduced to 1 $\mu M^{-1} s^{-1}$; the other rate constants were kept at the values given in Fig. 8b. The continuous curve is taken from c.

$K_{\text{eq}} = 6.1$ (Equation 2). The decrease of the binding amplitude between 10 and 200 s (Fig. 3b, black circles) reflects the $\text{cis} \leftrightarrow \text{trans}$ re-equilibration in the folded protein. It gives an apparent rate constant (A) of 0.007 $s^{-1}$ and, with Equations 1 and 2, microscopic rate constants of $k_f = 0.00602 s^{-1}$ and $k_b = 0.00098 s^{-1}$ (Fig. 8b).

The rate constants $k_{13}$ and $k_{14}$ are derived from the experiments in Figs. 4 and 7. The increase in refolding amplitude (0.56 ± 0.05 V, relative to the amplitude observed when SH3N is unfolded) is a measure for the 86% molecules that contain $\text{cis}$ Pro-238 (Fig. 6c). In the presence of the C3G peptide, the corresponding increase of the refolding amplitude was smaller (0.27 ± 0.02 V; Fig. 7), indicating that the fraction of molecules with a $\text{cis}$ Pro-238 had decreased to (0.27/0.56) × 86% = 41% in the protein-peptide complex. The shift of the $\text{cis}/\text{trans}$ equilibrium constant to 41/59 = 0.69 caused by ligand binding occurred with an apparent rate constant (A) of 0.008 $s^{-1}$ (Fig. 4), giving the microscopic rate constants $k_{13} = 0.0032 s^{-1}$ and $k_{14} = 0.0048 s^{-1}$ (Fig. 8b).

**Calculated Reaction Profiles Fit the Experimental Data**—The kinetic scheme in Fig. 8a and the rate constants in Fig. 8b were used to calculate the time courses of the unfolded and folded species during the refolding of the chicken SH3N-SH3C two-domain protein (Fig. 9a). In 89% of the unfolded molecules, Pro-238 is $\text{trans}$, and in 11% it is $\text{cis}$. $U_{\text{trans}}$ and $U_{\text{cis}}$ (dashed lines) are converted to $N_{\text{trans}}$ and $N_{\text{cis}}$ (continuous lines) within ~3 s. In the subsequent slow reaction, most $N_{\text{trans}}$ molecules are converted to $N_{\text{cis}}$, because the $\text{cis}$ form of Pro-238 is favored in the folded state. To examine whether the model in Fig. 8 provides an adequate description of the coupling between folding, Pro-238 isomerization, and function of chicken c-CrkII, we used the time-dependent distribution of species in Fig. 9a to calculate the kinetics of two crucial experiments.

First, the refolding kinetics probed by the binding of the C3G peptide were modeled. In the kinetic measurement (Fig. 3b), we could not discriminate between binding of the C3G peptide to the $\text{cis}$ and the $\text{trans}$ species. Therefore, we compared them with the sum of the calculated time traces for $N_{\text{cis}}$-$L$ and $N_{\text{trans}}$-$L$ (Fig. 9b). The observed fast increase in the binding amplitude largely reflects the rapid folding of the 89% $U_{\text{trans}}$ molecules to $N_{\text{trans}}$ (Fig. 9a), the high affinity form of SH3N-SH3C. The calculated time course for $N_{\text{cis}}$-$L + N_{\text{trans}}$-$L$ reproduces the experimental results well. In the slow reaction after folding, the $\text{cis}/\text{trans}$ equilibrium at Pro-238 is shifted toward the low affinity $\text{cis}$ form, $N_{\text{cis}}$, and thus leads to a decrease of the binding amplitude. The calculated time course for this decrease also agrees well with the experimental data (Fig. 9b).

For a second comparison, we calculated the kinetics of the Pro-238-limited binding reaction in Fig. 4. It originates from the shift of the $\text{cis}/\text{trans}$ equilibrium at Pro-238 in the folded protein ($N_{\text{cis}} \equiv N_{\text{trans}}$; Fig. 8) caused by the C3G peptide, which binds preferentially to $N_{\text{trans}}$. Again, the experimental data were well reproduced by the calculated curve (Fig. 9c). The amplitude profile of this reaction depends critically on the difference in substrate affinity between $N_{\text{cis}}$ and $N_{\text{trans}}$. As long as the peptide concentration is below the $K_D$ of $N_{\text{cis}}$ ($\approx 3.6 \mu M$), the high affinity $N_{\text{trans}}$ form ($K_D \approx 0.5 \mu M$) interacts preferentially with the ligand, and the equilibrium therefore is progressively shifted toward $N_{\text{trans}}$ giving rise to the observed slow proline-coupled binding reaction. With further increase of the ligand concentration, the low affinity form $N_{\text{cis}}$ starts to interact...
with the ligand as well, and the extent of additional binding limited by Pro-238 isomerization decreases.

To explore the sensitivity of our proposed reaction mechanism, we decreased the affinity of N\textsubscript{cis} for the C3G peptide 4-fold, by lowering \( k_0 \) from 16 to 4 \( \mu \text{M}^{-1} \text{s}^{-1} \) (Fig. 8) and then recalculated the amplitude profile (Fig. 9d). In this calculation, \( N_{\text{cis}} \) and \( N_{\text{trans}} \) differed 15-fold in affinity, instead of 3.7-fold. The calculation produces a much stronger shift in the cis/trans equilibrium with increasing peptide concentration than observed experimentally, and the maximum is shifted to 10 \( \mu \text{M} \) C3G peptide. Apparently, the experiment in Fig. 4 is highly sensitive toward the relative substrate affinities of the open \textit{trans} Pro-238 and the closed \textit{cis} Pro-238 forms. The strong deviation observed in Fig. 9d, as well as the good agreement between the experimental and calculated kinetic curves in Fig. 9b, c indicates that the four-species scheme in Fig. 8 and the rate constants in Fig. 8b provide an adequate description for the interrelationship between protein folding, Pro-238 native state cis/trans isomerization, and ligand binding in chicken c-CrkII.

**DISCUSSION**

In its function as a signal adapter, c-CrkII employs a domain opening/closing reaction between its two SH3 domains to control signal propagation from oncogenic tyrosine kinases to downstream target proteins. In the chicken but not in the human protein, the opening/closing reaction is coupled with cis/trans isomerization at Pro-238 in the SH3C domain. For the regulation of domain interactions by the isomeric state of a prolyl bond, an energetic linkage must exist between the two processes, i.e. a cis Pro-238 must lead to additional interdomain interactions, which stabilize the closed form and shift the equilibrium at Pro-238 toward the intrinsically unfavorable cis form.

Kinetic double-mixing experiments revealed how the folding of SH3N-SH3C, the cis/trans equilibrium at Pro-238 and the binding of the SH3N domain to a downstream ligand are mutually interdependent. In the closed form with a cis Pro-238, the two SH3 domains are not firmly locked. This explains why the binding site of the SH3N domain retains a low affinity for its ligand. In c-CrkII, prolyl isomerization thus does not switch between a domain-open and a domain-locked form, as in the gene-3 protein of a filamentous phage (20, 21), but between a high affinity and a low affinity form.

The kinetic double-box model in Fig. 8 describes the energetic linkages between folding, Pro-238 isomerization, and ligand binding in a quantitative fashion. During folding, the fraction of molecules with a cis Pro-238 increases from 11 to 86%, which requires ~9 kJ/mol of conformational energy. The energetic coupling between conformational folding and Pro-238 isomerization occurs presumably in two steps. In the folded SH3C domain, the cis content at Pro-238 is already increased to 40~50%, as suggested by the NMR structures and the folding kinetics of the isolated domain (9, 11). The assembly with SH3N in the domain closing reaction then increases the cis content further to 86%, which requires ~5 kJ/mol of conformational energy. The formation of one or two extra hydrogen bonds would be sufficient to provide this energy (22). Interestingly, ligand binding shifts the cis/trans equilibrium back to ~40/60, similar to the ratio observed for the isolated SH3C domain.

Pro-238 isomerization in chicken c-CrkII does not function as a simple on/off switch. The switching is not between almost all-cis and almost all-trans, and in the cis form the binding activity is not completely abolished but only reduced. Incomplete switching could be a consequence of working \textit{in vitro} with a purified two-domain fragment of c-CrkII. Alternatively, it may indicate that for a central regulator molecule such as c-CrkII, simple on/off switching is not adequate, because it impedes the fine-tuning of signaling. We view c-CrkII as an allosteric system consisting of an open high affinity R state and a closed low affinity T state. The coupling of the T \( \rightarrow R \) transition with prolyl isomerization would provide it with a memory. It slows the T \( \rightarrow R \) transition during activation, and after cis \( \rightarrow \text{trans} \) isomerization the protein remains in the high affinity R state for an extended time even when the concentration of an activating ligand has decreased again. In combination, this would result in a smoothing of the response curve, the extent of which could be controlled by prolyl isomerases. Native state prolyl isomerization thus might function not only as a molecular memory but also as a molecular attenuator in the regulation of protein function.

C-CrkII is very well studied, but the involvement of Pro-238 isomerization into the regulation of the chicken protein was detected only recently by biophysical \textit{in vitro} approaches. It remains to be determined how this isomerization modulates the cellular functions of c-CrkII.

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