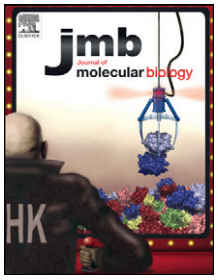

Long-Range Energetic Changes Triggered by a Proline Switch in the Signal Adapter Protein c-CrkII

Philipp A.M. Schmidpeter, Lena K. Ries, Tatjana Theer and Franz X. Schmid

Laboratorium für Biochemie und Bayreuther Zentrum für Molekulare Biologie, *Universität Bayreuth, 95440 Bayreuth, Germany*

Correspondence to Franz X. Schmid: *Biochemisches Laboratorium, Universität Bayreuth, 95440 Bayreuth, Germany.*
fx.schmid@uni-bayreuth.de
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Abstract

The signal adapter protein c-CrkII from chicken but not from human uses isomerization at Pro238 in the SH3C domain to regulate the activity of the SH3N domain. The different behavior of human and chicken c-CrkII originates from only two differences in sequence, at positions 239 after Pro238 and 272 in the N-Src loop of SH3C. We analyzed the kinetics of substrate binding to SH3N and an assay for its coupling with Pro238 isomerization in SH3C to identify the molecular path from Pro238 to the substrate binding site of SH3N. The *trans* → *cis* isomerization at Pro238 and a relocation of Phe239 re-organize the energetics of a hydrophobic cluster in the N-Src loop of SH3C and re-shape this region to optimize its interactions with SH3N. Concomitantly, the backbone becomes strained at Met272. We suggest that, in human c-CrkII, movement at position 239 and strain at position 272 are not tolerated because the β-branched residues Ile239 and Val272 restrain the backbone mobility and thus destabilize the *cis* Pro238 form.

Introduction

Cis/*trans* isomerizations of peptidyl-prolyl bonds are intrinsically slow reactions and constitute rate-limiting steps in the folding of many proteins [1–3]. They can serve also as slow molecular switches or timers in the regulation of protein function [4–11]. The signal adapter protein c-CrkII (cellular CT10 regulator of kinase) from chicken contains such a regulatory proline switch [12–20]. In c-CrkII, an N-terminal SH2 domain is followed by two SH3 domains (SH3N and SH3C), which are connected by a long linker of about 40 residues (Fig. 1) [16,17]. c-CrkII is a central regulatory hub in signaling networks and links oncogenic tyrosine kinases with cellular downstream partners. Deregulated forms of this protein promote tumor progression [21]. Two types of regulation are known for c-CrkII: inhibitory tyrosine phosphorylation [22] and native-state prolyl isomerization [16,18]. Non-receptor tyrosine kinases, such as c-Abl, phosphorylate Tyr222 in the linker between the SH3 domains. Phospho-Tyr222 then binds intramolecularly to the SH2 domain and thereby inhibits signal uptake and propagation [22–26]. A secondary phosphorylation at Tyr252 has a *trans*-activating effect on c-Abl [27–29].

The other regulatory mechanism involves *cis*/*trans* isomerization at Pro238, which is located at the transition between the inter-domain linker and the SH3C domain (Fig. 1). Chicken c-CrkII adopts an open, signaling-active conformation (R state) when Pro238 is *trans*. With *cis* Pro238, the SH3C domain interacts with SH3N, leading to a closed, inactive form (T state), in which access to the ligand binding site of the SH3N domain is obstructed (Fig. 1) [16,17]. Various shortened constructs of c-CrkII have been analyzed *in vitro*. In the SH3C domain, extended by the C-terminal part of the inter-domain linker, the *cis* and *trans* Pro238 forms are about equally populated. In the SH3N-SH3C two-domain protein, the *cis* content is increased to about 90% [17,30] apparently because inter-domain interactions provide additional conformational folding energy to further shift the *cis*/*trans* equilibrium toward the intrinsically unfavorable *cis* Pro238. These domain interactions are highly dynamic, and thus, the closed state is not an inactive but rather is a low-affinity state [18,30]. Ligand binding to SH3N

opens the domains and shifts the equilibrium toward th high-affinity state with *trans* Pro238. Linking the R → T transition with a slow prolyl *trans*-to-*cis* isomerization endows c-CrkII with a built-in memory or a molecular attenuator during signal transduction [30].

Such a mechanism based on native-state prolyl isomerization was observed for chicken but not for human c-CrkII [25,31]. This difference could be traced back to the amino acids at positions 239 and 272 in the SH3C domain [18]. Phe239 becomes exposed upon *trans* → *cis* isomerization at Pro238 and is thus able to contact the SH3N domain [17]. Met272 is remote from Pro238 and resides at a solvent-exposed position in the N-Src loop of SH3C (Fig. 1). Its importance for the Pro238-regulated inter-domain communication in c-CrkII was therefore unexpected. In human c-CrkII, the simultaneous substitutions of Ile239 and Val272 by Phe and Met, respectively, were necessary and sufficient to establish a regulatory prolyl isomerization as in the chicken protein [18].

In the present study, we used this I239F V272M variant of the human two-domain protein SH3N-SH3C (SH3N-SH3C$_{FM}$) as the starting molecule for a mutational analysis. It is closest in sequence to human c-CrkII but shows regulatory prolyl isomerization as the chicken protein. The aim was to uncover the energetic interactions that translate the *cis*/*trans* switching at Pro238 into a changed intramolecular affinity of SH3C for SH3N. In particular, we focused on the impact of the inter-domain linker (residues 230–237) and on the molecular path that links the isomeric state of Pro238 with Met272 within the SH3C domain. We probed the accessibility of the ligand binding site on SH3N of the variants by measuring the kinetics of substrate association. We also examined how, in a reciprocal fashion, substrate binding changes the *cis*/*trans* equilibrium at Pro238. This analysis revealed how critical residues of the linker and of the N-Src loop of SH3C communicate the isomeric state of Pro238 within and between the domains of c-CrkII.

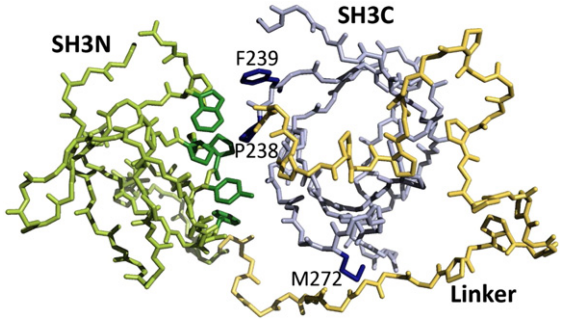

Fig. 1. Structure of the SH3N-SH3C two-domain fragment of chicken c-CrkII. SH3N and SH3C are shown in light green and light blue, respectively. The side chains of SH3N residues involved in substrate binding are shown in dark green, and the side chains of Pro238, Phe239, and Met272 are shown in dark blue. The inter-domain linker is shown in yellow. The figure was created using Protein Data Bank file 2L3S [17] and PyMOL [39].

Results

Energetic interaction between SH3C and the inter-domain linker

The SH3N and SH3C domains of c-CrkII are connected by a long linker of approximately 40 residues. It consists of a variable and flexible N-terminal part and a conserved and ordered C-terminal part, which is in physical contact with SH3C (Fig. 2a and b) [16,17]. To identify energetic interactions of SH3C with the linker, we produced variants of different chain lengths and determined their stabilities. The variants are labeled by their first residue (e.g., variant 234-SH3C begins with Leu234; Fig. 2c). For all of them, GdmCl-induced equilibrium unfolding transitions and the kinetics of unfolding and refolding were measured. The comparison of the 237-SH3C variant of human c-CrkII (which lacks the linker; Fig. 2d) and the variant 200-SH3C (with the full linker) indicates that the linker stabilizes the SH3C domain and shifts the midpoint of its unfolding transition from 1.4 M to 1.7 M GdmCl (Table 1). This modest increase in stability originates from a decrease in the rate of unfolding (Fig. 2e), which suggests that stabilizing interactions between the linker and SH3C are established late in folding after passage over the transition state of folding. Similar results were obtained for SH3C from chicken (Fig. S1 and Table 1).

The first part of the linker (residues 200–229) is apparently unimportant for the stability of SH3C (Table 1 and Fig. S1c). The stabilizing interactions between the linker and SH3C must thus originate from residues in the 230-237 region. To identify these residues, we started with 237-SH3C and successively lengthened the linker. The stepwise extension down to Asn233 affected neither the stability nor the folding kinetics (Fig. 3a and b, Table 1, and Table S1). Minor differences in the equilibrium unfolding transitions probably originated from the short base lines for the native protein.

Adding four more residues (in the variant 229-SH3C) led to the full extent of stabilization as observed for 200-SH3C (Fig. 3a, Fig. S1c, Table 1, and Table S1). The 229-233 region contains the motif Pro230-Leu231-Pro232 (Fig. 2c). The substitutions of Pro230 and Pro232 by Ala (229-SH3C P230A P232A) had similar effects on the unfolding transition (Fig. S2a) and the folding kinetics