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Ligand-linked stability of mutants of the C-domain of calmodulin

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Abstract

There is a necessary energetic linkage between ligand binding and stability in biological molecules. The critical glutamate in Site 4 was mutated to create two mutants of the C-domain of calmodulin yielding E140D and E140Q. These proteins were stably folded in the absence of calcium, but had dramatically impaired binding of calcium. We determined the stability of the mutant proteins in the absence and presence of calcium using urea-induced unfolding monitored by circular dichroism (CD) spectroscopy. These calcium-dependent unfolding curves were fit to models that allowed for linkage of stability to binding of a single calcium ion to the native and unfolded states. Simultaneous analysis of the unfolding profiles for each mutant yielded estimates for calcium-binding constants that were consistent with results from direct titrations monitored by fluorescence. Binding to the unfolded state was not an important energetic contributor to the ligand-linked stability of these mutants.

Keywords: Ligand binding; Thermodynamic linkage; Calcium binding; Unfolded state; Partition function

1. Introduction

Ligand binding is an important factor in mediating biological activity of many macromolecules. Because of the propagated effects of ligand binding in macromolecules, there have been a number of studies that explore ligand linkage in other relevant equilibria such as stability, dimerization, and conformational change. This is the basis for several studies of the linkage of stability to proton binding [1,2], ion binding [3,4], and binding of cytidine-2′-monophosphate to Ribonuclease A [5]. It has been applied to the influence of ligands on conformational state [6–8] and dimerization [9]. The theoretical foundations have been laid for studying the relationship between ligand binding with different affinities to different states of a macromolecule, thereby influencing the position of equilibrium between these states [10,11]. Our laboratory is interested in the linkage between ligand binding and stability in calcium-binding proteins.

Calcium-binding proteins are well-suited to linkage studies. Calcium can be varied over a wide range of concentrations, and it does not interfere with typical spectroscopic signals from the protein. This paper reports linkage studies of mutants of the C-terminal domain of calmodulin in which Site 4 has been disabled by mutation of the terminal glutamate. These proteins provide excellent models with which to explore the linkage outlined in Scheme 1 and offer the following advantages.

(1) The proteins bind one ligand to the native state with moderate affinity that can be easily resolved from any low affinity events.
(2) The apo-proteins in the native state are folded to allow for measurement of the unfolding free energy in an independent experiment.
(3) The proteins have intrinsic spectroscopic signals for monitoring secondary structure and calcium binding.

The apo-native state (N) binds one calcium ion according to Reaction 1 to create the calcium-saturated native state (N·Ca). Both the apo-and calcium-saturated native states can be unfolded (Reactions 2 and 3) to form the apo-

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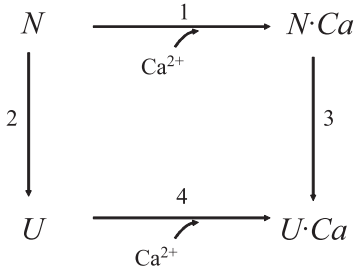

Scheme 1. Linkage of ligand binding to stability considering binding to unfolded state.

unfolded state (U) and the calcium-saturated unfolded state (U · Ca). Reaction 4 considers the possibility that a calcium ion binds to the unfolded state. The experiments described in this paper test this simple relationship between the native and unfolded states as a function of added ligand. A focus of this study is to determine whether it is necessary to consider ligand binding to the unfolded state (to form U · Ca) in order to adequately describe linked processes over a range of conditions. Although the C-domain of calmodulin offers a convenient spectroscopic signal for binding calcium to the native state, the studies presented here provide an option for inferring the ligand-binding constants in systems where a spectroscopic signal for the binding event is not available. This approach may be particularly useful in measuring weak interactions or those in which the spectral signals are not equivalent for each binding event.

Calmodulin is the quintessential member of the EF-hand family of calcium-binding proteins. Vertebrate calmodulin has four functional calcium-binding sites, two in each of two domains. The N-terminal domain contains Sites 1 and 2 and the C-terminal domain contains Sites 3 and 4. Although the domains are separated by a long central helix, they are known to influence each other both in the absence [12–14] and presence of physiological targets [15]. The domains can be separated by cleavage in the central helix region, but not without consequence to the calcium-binding properties of the sites in each domain [16–18]. Isolated N- and C-terminal domains are folded; they also bind calcium [16,17,19,20].

Foundations for this work were laid in a comprehensive study of the calcium-dependent stability of wild-type calmodulin and its isolated domains by Masino et al. [17]. The work reported here extends the studies of wild-type calmodulin in two important ways. Firstly, we bring to fruition the concepts introduced in that work to allow resolution of actual binding constants from unfolding data. This is important because it weaves theoretical and experimental approaches to allow characterization of each leg of the thermodynamic cycle depicted in Scheme 1. Secondly, we extend the work of Masino et al to include consideration of binding site mutants that bind only one ligand at mM levels of calcium, but have similar stabilities to wild-type. Consideration of only one ligand simplifies the analysis of the data allowing estimates of binding constants from unfolding data.

The proteins reported in this study are mutants of the isolated C-terminal domain of calmodulin (residues 76–148). The C-domain contains two EF-hand calcium-binding sites (Fig. 1A), Sites 3 and 4. In each of the mutants, Site 4 is impaired by mutation of the critical glutamate in position 12 (E140; Fig. 1B) to either glutamine or aspartate. Thus, we have created small, predominantly helical proteins that are soluble and bind nominally one calcium ion with moderate affinity to examine the thermodynamic linkage outlined in Scheme 1. This paper describes stability studies for E140D and E140Q, two mutants of the C-domain of calmodulin in the absence and presence of calcium. Calcium-binding constants for the native state, resolved from the unfolding studies, are compared to those determined independently in fluorescence experiments.

2. Experimental

2.1. Cloning

The expression plasmid for the C-domain of wild-type rat calmodulin (residues 76–148; denoted WT C-domain) was a kind gift of M. A. Shea (U. of Iowa). The gene was cloned into a pET7-7 vector between *Nde*I (5′end of gene) and *Bam*HI (3′end of gene) restriction enzyme sites [16]. Mutants were created using the Quickchange Mutagenesis Kit from Stratagene (LaJolla, CA). The instructions for the creation of mutagenic primers that were provided with the kit were followed to create two pairs of complimentary primers. Primers were synthesized, purified, and quantitated

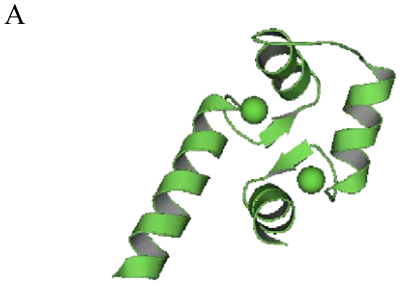

Fig. 1. Structure and sequence of C-domain of calmodulin. (A) Ribbon drawing of the Ca^{+2}-saturated C-terminal domain of calmodulin. The Cα-backbone of residues 76–148 from 3cln.pdb [31] are illustrated as a ribbon using PyMol (DeLano Scientific LLC; http://www.pymol.org). Calcium ions in each site are shown. Site 3 is to the left and Site 4 to the right. (B) Amino acid sequence of the C-domain of calmodulin from residues 76 to 148. The 12 residues of the calcium-binding site are highlighted (Site 3=residues 93–104; Site 4=residues 129–140) and aligned. The terminal glutamate (12th position), E140, is underlined and is mutated to either D or Q in the proteins studied here.