doi:10.1016/j.jmb.2010.02.014 J. Mol. Biol. (2010) 397, 991–1002

JMB

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Structure of Avian Thymic Hormone, a High-Affinity Avian β-Parvalbumin, in the Ca^{2+}-Free and Ca^{2+}-Bound States

Jonathan P. Schuermann[1], Anmin Tan[2], John J. Tanner[2,3] and Michael T. Henzl[2]*

[1]*Northeastern Collaborative Access Team (NE-CAT), Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA*

[2]*Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, MO 65211, USA*

[3]*Department of Chemistry, University of Missouri, Columbia, MO 65211, USA*

Received 25 November 2009; received in revised form 28 January 2010; accepted 9 February 2010
Available online 12 February 2010

Originally isolated on the basis of its capacity to stimulate T-cell maturation and proliferation, avian thymic hormone (ATH) is nevertheless a parvalbumin, one of two β-lineage isoforms expressed in birds. We recently learned that addition of Ca^{2+}-free ATH to a solution of 8-anilinonaphthalene-1-sulfonate (ANS) markedly increases ANS emission. This behavior, not observed in the presence of Ca^{2+}, suggests that apolar surface area buried in the Ca^{2+}-bound state becomes solvent accessible upon Ca^{2+} removal. In order to elucidate the conformational alterations that accompany Ca^{2+} binding, we have obtained the solution structure of the Ca^{2+}-free protein using NMR spectroscopy and compared it to the Ca^{2+}-loaded protein, solved by X-ray crystallography. Although the metal-ion-binding (CD–EF) domains are largely coincident in the superimposed structures, a major difference is observed in the AB domains. The tight association of helix B with the E and F helices in the Ca^{2+}-bound state is lost upon removal of Ca^{2+}, producing a deep hydrophobic cavity. The B helix also undergoes substantial rotation, exposing the side chains of F24, Y26, F29, and F30 to solvent. Presumably, the increase in ANS emission observed in the presence of unliganded ATH reflects the interaction of these hydrophobic residues with the fluorescent probe. The increased solvent exposure of apolar surface area in the Ca^{2+}-free protein is consistent with previously collected scanning calorimetry data, which indicated an unusually low change in heat capacity upon thermal denaturation. The Ca^{2+}-free structure also provides added insight into the magnitude of ligation-linked conformational alteration compatible with a high-affinity metal-ion-binding signature. The exposure of substantial apolar surface area suggests the intriguing possibility that ATH could function as a reverse Ca^{2+} sensor.

Keywords: calcium-binding protein; EF-hand protein; parvalbumin; NMR structure; crystal structure

Edited by R. Huber

Introduction

The second-messenger role of Ca^{2+} in eukaryotic signal transduction pathways is largely mediated by EF-hand proteins.[1–4] The human genome, for example, encodes 242 EF-hand family members.[5] Some of these, calmodulin being the archetype, have explicit regulatory activity, modulating the activities of effector proteins in a Ca^{2+}-dependent manner. Other EF-hand proteins function as mobile intracellular Ca^{2+} buffers. Regardless of their precise role, all display the hallmark Ca^{2+}-binding motif—a central ion-binding loop flanked by short amphi-

*Corresponding author. E-mail address: henzlm@missouri.edu.

Abbreviations used: ANS, 8-anilinonaphthalene-1-sulfonate; ATH, avian thymic hormone; EDTA, ethylenediaminetetraacetic acid; HSQC, heteronuclear single quantum coherence; Mes, 4-morpholineethanesulfonic acid; NOE, nuclear Overhauser effect; PV, parvalbumin; PDB, Protein Data Bank; NE-CAT, Northeastern Collaborative Access Team; TOCSY, total correlated spectroscopy.

pathic helical segments. The term "EF-hand" was inspired by the recognition that the spatial arrangement between these structural elements can be mimicked with the fingers of the right hand.[6]

Despite the general similarity of their metal-ion-binding sites, EF-hand proteins exhibit broad variations in divalent ion affinity. We are exploring the physical and structural basis for these differences, using specific parvalbumin (PV) isoforms. PVs are small (M_r=12,000), vertebrate-specific EF-hand proteins.[3,7,8] The PV family includes α- and β sublineages, distinguished by isoelectric point (pI>5 for α) and lineage-specific sequence differences.[9,10] Mammals express two isoforms (one from each lineage[11]) that exhibit 49% sequence identity. Despite the sequence similarity, in 0.15 M NaCl at pH 7.4, rat α-PV binds Ca^{2+} with a standard free energy change 3.5 kcal mol^{-1} more favorable than that of rat β-PV.[12] Whereas Na^+ competes, albeit weakly, for vacant EF-hand sites in both proteins, only the β isoform binds K^+. Thus, when K^+ replaces Na^+ as the major solvent cation, the α isoform experiences an apparent increase in divalent ion affinity, binding Ca^{2+} a full 5.5 kcal mol^{-1} more tightly than rat β-PV. Besides improving our understanding of this biologically important class of protein, an explanation for the disparity in binding affinity could furnish insight into protein–ligand interactions in general.

The Protein Data Bank (PDB) contains more than 20 high-resolution structures of Ca^{2+}-bound PVs—including carp β (5CPV),[13] leopard-shark α (5PAL),[14] pike β (2PVB),[15] rat α (1RWY),[16] and rat β (1RRO).[17] Their overall structural similarity—average RMSD< 1.0 Å for backbone atoms—suggested that variations in divalent-ion-binding affinity might, in fact, reflect differences in the structures of the apoproteins. Accordingly, solution structures were obtained for the Ca^{2+}-free rat α- and β-PVs.[18,19] Ca^{2+} removal from the β isoform evidently provokes a substantial conformational rearrangement, implying that the attenuated divalent ion affinity may reflect the energetic cost associated with isomerizing the apoprotein. If correct, then Ca^{2+} binding should be accompanied by more muted structural alterations in high-affinity isoforms. That idea is supported by solution structural data for the Ca^{2+}-free rat α isoform, which closely resembles the Ca^{2+}-bound protein. Together, these findings implied a direct correlation between binding affinity and conformational similarity of the Ca^{2+}-free and Ca^{2+}-loaded states. The Ca^{2+}-free PV structure described herein suggests that the correlation is not quite that straightforward.

Avian thymic hormone (ATH) was originally isolated on the basis of, and named for, its capacity to stimulate avian T-cell maturation and proliferation.[20,21] Quite unexpectedly, sequence analysis of the purified protein revealed that it was a β-PV.[22] In fact, ATH is one of two β-PV isoforms expressed in chicken thymus tissue. The other is known as chicken PV 3,[23] also believed to serve an endocrine role in the avian immune system.[24] Both EF-hand sites in ATH qualify as high-affinity, or Ca^{2+}/Mg^{2+}, sites. The Ca^{2+}-binding constants in 0.15 M NaCl, at pH 7.4, are 2.4×10^8 and 1.0×10^8 M^{-1}; the corresponding Mg^{2+} constants are 2.2×10^4 and 1.2×10^4 M^{-1}.[25,26]

We recently learned that addition of Ca^{2+}-free ATH to a solution of the hydrophobic probe 8-anilinonaphthalene-1-sulfonate (ANS) produces a large increase in fluorescence quantum yield and a pronounced blue shift.[27] Addition of the Ca^{2+}-bound protein is without effect. This behavior, which is not observed with the Ca^{2+}-free forms of either rat α-PV or chicken PV 3, implies that the conformations of the Ca^{2+}-free and Ca^{2+}-bound protein differ substantively. This finding conflicts with the proposed correlation between divalent ion affinity and conformational similarity of the Ca^{2+}-free and Ca^{2+}-loaded states of high-affinity PV isoforms. In an effort to resolve this issue and to delineate the conformational changes that occur in ATH upon Ca^{2+} binding, we have determined the solution structure of Ca^{2+}-free ATH. To permit a more meaningful evaluation of the structural changes provoked by Ca^{2+} removal, we also report the crystal structure of Ca^{2+}-bound ATH.

Results

Ca^{2+}-bound ATH

The structure of Ca^{2+}-bound ATH was solved by X-ray crystallography (Table 1). The crystal morphology (long needles) and space group (P1), its sensitivity to radiation damage, and the unit cell dimensions (containing eight copies of the protein) made this task challenging. Ultimately, the experimental hurdles were overcome by employing the micro-diffraction beamline at Northeastern Collaborative Access Team (NE-CAT) 24-ID-E of the Advanced Photon Source. The structure was solved to a resolution of 1.95 Å, using a wedge-based data collection strategy, featuring a wide oscillation width of 4° per frame, as described in Materials and Methods.

Ca^{2+}-bound ATH displays the characteristic PV fold, consisting of six α-helices (labeled A–F in Fig. 1a) organized into two domains. The AB domain, spanning residues 1–38, includes the A and B α-helices and an extended loop. The remaining polypeptide chain forms the CD/EF domain, which includes the two EF-hand metal-ion-binding motifs. The eight chains in the asymmetric unit of the crystal have very similar structures. Pairwise RMSDs for C^α atoms in the eight chains span the range 0.34–0.93 Å, with an average of 0.61 Å.

Ca^{2+}-free ATH

Resonance assignments

Assignments were made using a series of triple-resonance experiments. CBCACONH and HNCACB spectra yielded tentative assignments for nearly all