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Comprehensive analysis of the roles of 'black' and 'gray' clusters in structure and function of rat β-parvalbumin

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ABSTRACT

Recently we found two highly conserved structural motifs in the proteins of the EF-hand calcium binding protein family. These motifs provide a supporting scaffold for the Ca^{2+} binding loops and contribute to the hydrophobic core of the EF-hand domain. Each structural motif forms a cluster of three amino acids called cluster I ('black' cluster) and cluster II ('grey' cluster). Cluster I is much more conserved and mostly incorporates aromatic amino acids. In contrast, cluster II includes a mix of aromatic, hydrophobic, and polar amino acids. The 'black' and 'gray' clusters in rat β-parvalbumin consist of F48, A100, F103 and G61, L64, M87, respectively. In the present work, we sequentially substituted these amino acids residues by Ala, except Ala100, which was substituted by Val. Physical properties of the mutants were studied by circular dichroism, scanning calorimetry, dynamic light scattering, chemical crosslinking, and fluorescent probe methods. The Ca^{2+} and Mg^{2+} binding affinities of these mutants were evaluated by intrinsic fluorescence and equilibrium dialysis methods. In spite of a rather complicated pattern of contributions of separate amino acid residues of the 'black' and 'gray' clusters into maintenance of rat β-parvalbumin structural and functional status, the alanine substitutions in the cluster I cause noticeably more pronounced changes in various structural parameters of proteins, such as hydrodynamic radius of apo-form, thermal stability of Ca^{2+}/Mg^{2+}-loaded forms, and total energy of Ca^{2+} binding in comparison with the changes caused by amino acid substitutions in the cluster II. These findings were further supported by the outputs of computational analysis of the effects of these mutations on the intrinsic disorder predisposition of rat β-parvalbumin, which also indicated that local intrinsic disorder propensities and the overall levels of predicted disorder were strongly affected by mutations in the cluster I, whereas mutations in cluster II had less pronounced effects. These results demonstrate that amino acids of the cluster I provide more essential contribution to the maintenance of structuraland functional properties of the protein in comparison with the residues of the cluster II.

Abbreviations: CD, circular dichroism spectroscopy; DSC, differential scanning calorimetry; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis2-aminoethylether-*N*,*N*,*N*',*N*'-tetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid); ME, 2-mercaptoethanol; PA, parvalbumin; β-PA, β isoform of parvalbumin; intact PA, parvalbumin isolated from muscles; rWT β-PA, recombinant wild-type α-parvalbumin; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl) amino methane

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1. Introduction

Calcium-binding proteins play important roles in practically all biological processes. Based on their structural and functional peculiarities, calcium-binding proteins are grouped in several large families (for review see, for example, [1–3]). One of the most studied families of calcium-binding proteins is the EF-hand protein family (reviewed in [3]). The EF-hand calcium-binding domain is a structural and functional unit that consists of two helices and a calcium binding loop between them. This domain was first found in carp parvalbumin (pI 4.25) by Kretsinger et al. in 1972 [4–6]. Most of the EF-hand family members, such as calmodulin and troponin C, function as Ca^{2+}-dependent sensor proteins. Others, mostly parvalbumin and calbindin, seem to serve as cytosolic Ca^{2+} buffers [1].

The EF-hand domains in Ca^{2+}-binding proteins are usually paired. Examination of the representative tertiary structures of the EF-hand containing proteins from eleven structural subfamilies enabled us to reveal structural nonequivalence of individual EF-hands in the paired EF-hand domains [7]. In fact, as a result of comprehensive comparison, we found two highly conserved structural motifs, which provide a supporting scaffold for the Ca^{2+} binding loops and contribute to the hydrophobic core of the EF-hand domain. Each structural motif contains a cluster of three amino acids. These clusters were called cluster I ('black' cluster) and cluster II ('grey' cluster). Cluster I ('black') is much more conserved in the various members of the EF-hand protein family and mostly incorporates aromatic amino acids. It lacks destabilizing interactions and has a predominant aromatic mini-core that is stabilized by a set of linked CH-π and CH—O hydrogen bonds. Based on these observations, we suggested that cluster I is likely vital for structural stabilization of the EF-hand domain in its critical gate region, where the polypeptide chain enters and exits the domain [7].

In contrast, cluster II includes a mix of aromatic, hydrophobic, and polar amino acids of different sizes. It is much less conserved and lacks stabilizing interactions and contain residues that are more often engaged in destabilizing interactions. We suggested that the higher variability of cluster II ('gray') could promote adaptation of an EF-hand domain to the conformational and dynamic requirements imposed by the need to ensure wide range of kinetic and equilibrium metal binding constants, as well as recognition of various targets (proteins, lipids and so on) [7].

The analysis of the structures of clusters I and II and examination of their rearrangements in response to Ca^{2+} binding enabled us to propose a more detailed classification of the EF-hand proteins, which was different from the classical division of such proteins on metal sensors and calcium buffers [7]. Using a group of α- and β-parvalbumins as illustrative representatives of the EF-hand protein family, we investigated the relationship between the location of the 'black' and 'gray' structural clusters and local intrinsic disorder predisposition of these proteins [8]. This analysis showed that residues the vicinity of the 'black' and 'gray' structural clusters are enriched in the disorder-promoting residues, suggesting the presence of conserved structural dynamics in parvalbumins [8]. However, at the global level, structural and functional significance of the 'black' and 'grey' clusters in calcium-binding proteins remains to be elusive. The goal of the present work was to fill this gap in current knowledge. To this end, we conducted systematic experimental studies of the roles of the 'black' and 'grey' clusters in rat β-parvalbumin.

Parvalbumin (reviewed by Permyakov [9]) is a member of the EF-hand family of calcium binding proteins. It is a small (Mr 10.5–12 kDa), acidic (pI 3.9–6.6), cytosolic Ca^{2+}-binding protein, found in lower and higher vertebrates, including humans (for reviews see [9–12]). Parvalbumin is present in fast-twitch muscle cells, specific neurons of the central and peripheral nervous system, certain cells of several endocrine glands, and sensory cells of the mammalian auditory organ, the organ of Corti, and some other cells. The highest concentration of parvalbumin (up to several millimoles per liter) was found in fast muscles (mainly in skeletal, but sometimes in cardiac) [13,14]. Parvalbumin serves as a soluble relaxing factor accelerating Ca^{2+}-mediated relaxation phase in fast muscles. This functional model is supported by the direct gene transfer experiments [15] and by studies of mice with knockout parvalbumin gene [16]. The exact functions of this protein in nerve cells are still unknown, nevertheless it is assumed that its major role is connected with Ca^{2+} buffering, Ca^{2+} transport, and regulation of various enzyme systems [9].

Parvalbumins evolutionarily diverged into two distinct sub-lineages, α and β [17–19], differing in their isoelectric points (α: pI > 5; β: pI < 5), length of the C-terminal helix (1 residue longer in α), and substitutions of at least 11 amino acid residues. A cysteine at position 18 and an aspartic acid at position 61 are characteristic of β-parvalbumins [20]. Parvalbumin possesses two active EF-hand Ca^{2+} binding sites formed by the CD and EE loops with flanking α-helices (CD and EF domains, respectively) (reviewed in [9]). The AB domain of PA cannot bind Ca^{2+} due to the shortened AB-loop and disturbed Ca^{2+} binding DxDxDG motif. Meanwhile, the AB domain covers the hydrophobic surface of the functional EF-hands and modulates their metal affinities [21–23].

Fig. 1 shows the structure of rat β-PA (also known as oncomodulin) and demonstrates localization of the 'black' and 'gray' clusters, which include F48, A100, F103 and G61, L64, M87, respectively. In our work, we sequentially substituted the amino acids of the 'black' and 'gray' clusters by alanine and studied physical properties of the resulting mutant proteins. It turns out that, in spite of rather complicated pattern of contributions of separate residues of the clusters of rat β-parvalbumin to maintenance of the structural and functional properties of the protein, the changes in such parameters as hydrodynamic radius of apoform, thermal stability of Ca^{2+}/Mg^{2+}-loaded forms and total calcium binding energy, the alanine substitutions in the 'black' cluster (I) of β-parvalbumin result in essentially more pronounced effects in comparison with the analogous substitutions in the 'gray' cluster (II).

2. Materials and methods

2.1. Materials

Molecular biology grade HEPES, ultra-grade H_3BO_3 and BioUltra-grade glycine were from Calbiochem, Fluka and Sigma-Aldrich Co, respectively. Ultra-pure grade Tris and analytical grade PMSF were purchased from Amresco. Buffer grade MOPS was from AppliChem.

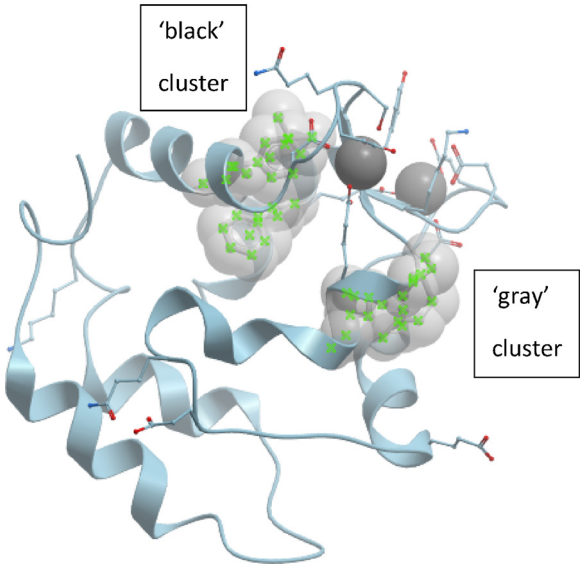

Fig. 1. Tertiary structure of Ca^{2+}-loaded (1RRO) rat β-PA. Bound calcium ions and residues of the 'black' and 'grey' clusters are shown.