A Proteasome Activator Subunit Binds Calcium*

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Claudio Realini and Martin Rechsteiner

From the Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, Utah 84132

We recently cloned a cDNA encoding the 29-kDa subunit of human red blood cell regulator (REG), a potent activator of the multicatalytic protease (Realini, C., Dubiel, W., Pratt, G., Ferrell, K., and Rechsteiner, M. (1994) J. Biol. Chem. 269, 20727–20732). The sequence of this subunit contains 28 “alternating” lysine and glutamic acid residues (a KEKE motif). Similar regions are present in a number of Ca²⁺-binding proteins, and using standard filter assays, the recombinant protein is shown to bind ⁴⁵Ca²⁺ and ruthenium red. ⁴⁵Ca²⁺ is also bound to a ubiquitin extension protein containing the 28-residue KEKE region from the 29-kDa REG subunit. Thus, the 29-kDa REG subunit is a Ca²⁺-binding protein, and its KEKE region is able to bind divalent cations. Ca²⁺ reversibly inhibits the enhanced peptidase activity of complexes between the multicatalytic protease and recombinant REG. This raises the possibility that multicatalytic protease activity is regulated by calcium in vivo.

The multicatalytic protease (MCP) or 20 S proteasome is a large (~700 kDa) multimeric enzyme found in eukaryotes, prokaryotes, and archaeabacteria (for reviews see Refs. 1–3). MCP subunits range in molecular mass from 20 to 30 kDa and can be placed in two families based on their homology to unique α- and β-subunits present in the archaeabacteria, Thermoplasma (4). The assembled enzyme consists of four stacked rings that form a hollow cylinder (5). The outer rings consist of 7 subunits of the α family, and the two inner rings each contain 7 β-type subunits (6, 7). It has been suggested that the enzyme’s active sites line a central aqueous channel (3), and the recent x-ray structure of MCP from Thermoplasma confirms this suspicion (8). Site-directed mutagenesis (9) and covalent labeling with a novel protease inhibitor (10) identify the N-terminal threonine residues of β-subunits as active site nucleophiles. Thus, MCP is the first example of a threonine protease.

In eukaryotes, MCP serves as the proteolytic core for two larger complexes. In an ATP-dependent reaction, MCP can associate with a regulatory protein complex that we call the regulator (REG), thereby producing a markedly activated peptidase (16, 17). REG binds to each end of MCP (18) and stimulates hydrolysis of selected fluorogenic peptides as much as 50-fold. SDS-polyacrylamide gel electrophoresis of regulator from human red blood cells revealed 2 subunits with apparent molecular masses of 31 and 29 kDa (17). We have recently cloned and expressed the gene for the 29-kDa subunit of human regulator (rREG₂₉K), and we have shown that the recombinant species activates MCP in a manner very similar to the molecule purified from human red blood cells (19).

A stretch of 28 alternating lysines and glutamate residues is a striking feature of the amino acid sequence of the 29-kDa subunit. We call such regions KEKE motifs and have proposed that these highly charged regions represent association domains (20); Perutz has also suggested that alternating positive and negative amino acids or “polar zippers” play a role in protein-protein association (21). KEKE motifs are found in a variety of proteins including subunits of the 26 S protease and MCP, microtubule-associated protein 1B, myosin phosphatase, triadin, and chaperonins such as hsp70 and hsp90 (20). They are also present in the calcium-binding proteins calreticulin, calnexin, endoplasmic, and Ca²⁺-dependent adenosine triphosphatase (Ca²⁺-ATPase). Calreticulin contains two distinct Ca²⁺-binding regions, one of which is a KEKE motif that binds calcium with high capacity and low affinity (22). Because KEKE motifs are present in Ca²⁺-binding proteins and because Ca²⁺ is an important regulator of cellular processes (23, 24), we asked whether the 29-kDa REG subunit is capable of binding Ca²⁺. Here we report that rREG₂₉K binds ⁴⁵Ca²⁺, ruthenium red, and the cationic dye, carbocyanine. Using recombinant technology, we show that, when appended to ubiquitin, the KEKE motif from rREG₂₉K confers Ca²⁺ binding to the chimeric protein. Furthermore, concentrations of Ca²⁺ in the micromolar range reversibly inhibit the peptidase activity of rREG₂₉K-MCP complexes.

EXPERIMENTAL PROCEDURES

Materials—Calf thymus was obtained from Boehringer Mannheim and ubiquitin from Sigma. The fluorogenic peptide succinyl-Leu-Leu-Val-Tyr (sLLVY-MCA) was obtained from Peninsula Laboratories. Ruthenium red and Stains All were from Spectrum Chemicals (New Brunswick, NJ); ⁴⁵Ca²⁺ (specific activity, 10 mCi/mg) was purchased from DuPont NEN.

Preparation of REG, Ubiquitin-KEKE, Ub-KEKE, and Multicatalytic Protease—Recombinant REG was purified to homogeneity from Escherichia coli cells induced with isopropyl-1-thio-β-D-galactopyranoside (19). Briefly, recombinant cells were lysed using a French press, and the cell lysate passed over an anion exchange column. Fractions from the peak of activity were pooled, and the regulator was further purified by size exclusion chromatography. The recombinant regulator was greater than 95% pure as determined by electrophoresis on a SDS-polyacrylamide gel followed by silver staining. Ub-KEKE(1-106) and Ub-KEKE(1-106) were expressed and purified according to published procedures (25). Multicatalytic protease was purified from outdated human red blood cells as described (26).

Fluorometric Protease Assays—Spectrofluorometric assays consisted of 100 μM sLLVY-MCA incubated in the presence of MCP and various amounts of recombinant REG in a final volume of 50–100 μl of 10 mM phosphate, pH 7.4. Reactions were initiated by the addition of fluoroo-
genic peptide and terminated by adding 200 μl of cold 100% ethanol. Fluorescence was measured on a Perkin-Elmer fluorometer using an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Imidazole acid was used to eliminate contaminating cations from protein solutions and buffers.

Binding of REG, Ub-KEKE, Ub-KEKE½, Ubiquitin, and Calmodulin to Ruthenium Red, Carboxyamine, and 45Ca²⁺—Purified rREG₂⁹K, ubiquitin, ubiquitin-KEKE fusion proteins, and calmodulin were applied to a nitrocellulose filter (0.2 μm, Schleicher and Schuell) using a slot blot apparatus and tested as described in Ref. 27 for their ability to bind 2 μM 45Ca²⁺ (1 μCi/ml) and ruthenium red (28) in the presence of 0.1, or 5 mM MgCl₂. Interaction of soluble proteins with the cationic carboxyamine dye, Stains All, was measured as described (29).

RESULTS

The 29-kDa REG Subunit Is a Calcium-binding Protein—There are three generally accepted methods for measuring calcium binding to proteins. Some calcium-binding proteins interact with the cationic carboxyamine dye, Stains All, and produce a characteristic absorption peak at 615 nm (29). Similarly, ruthenium red has been shown to bind many proteins capable of forming complexes with Ca²⁺ (29). Similarly, ruthenium red has been shown to bind many proteins capable of forming complexes with Ca²⁺ (29). Sim-

Fig. 1. Calcium binding by recombinant regulator. A, absorption spectra of Stains All in the presence of recombinant regulator (REG), calmodulin (CaM), and ubiquitin (Ub). Samples were 10 μg of protein in 1 ml of Tris pH 8.8, 0.001% Stains All, and 0.1% formamide. B, binding of regulator, Ub-KEKE½, Ub-KEKE, calmodulin, and ubiquitin to 45Ca²⁺ and ruthenium red (RR). The proteins (5 μg) were applied to a nitrocellulose membrane and probed with either ruthenium red (25 mg/ml) or 2 μM 45Ca²⁺ (1 μCi/ml) as described under “Experimental Procedures.” Filters were stained with Ponceau S to confirm that equal amounts of protein were bound in each slot.

ence presence of increasing concentrations of Mg²⁺ (Fig. 2). Whereas ubiquitin and bovine serum albumin failed to bind Ca²⁺ even in Mg²⁺-free solution, rREG₂⁹K, Ub-KEKE, and calmodulin bound Ca²⁺ in the presence of the competing cation. These results indicate that the 29-kDa REG subunit is a Ca²⁺-binding protein and that its KEKE motif is likely to confer this ability. Ub-KEKE½ contains the first 14 residues of the KEKE motif; nonetheless, it failed to bind 45Ca²⁺ in the presence of competing Mg²⁺ (Fig. 2). This suggests that either the entire KEKE motif is required for significant Ca²⁺ binding or that the second half of the KEKE motif (EDKDKKGGEDEKD) is responsible for interactions with Ca²⁺.

Effect of Ca²⁺ on the Activity of rREG₂⁹K-MCP Complexes—To address the relevance of Ca²⁺ binding to rREG₂⁹K, we assayed MCP peptidase activity in the presence of rREG₂⁹K, Ca²⁺, and EGTA. A standard peptidase assay was initiated and then rREG₂⁹K was added, followed several minutes later by Ca²⁺; EGTA was finally added after another several minutes. A typical spectrofluorometric trace is shown in Fig. 3A. Strong stimulation of peptidase activity was immediately observed upon addition of purified recombinant REG (Phase I → Phase II); peptide hydrolysis by the rREG₂⁹K-MCP complex was inhibited by Ca²⁺ (Phase III), but activity recovered after EGTA was added to chelate the Ca²⁺ (Phase IV). MCP alone did not respond to the addition of Ca²⁺ or EGTA (see Fig. 3B). The peptidase activity of rREG₂⁹K-MCP complexes was inhibited by Ca²⁺ in a dose-dependent manner (Fig. 3C); only 30% of the original activity remained at 300 μM Ca²⁺. The inhibitory effect of Ca²⁺ was not observed in the presence of EGTA, and the activity of MCP was only minimally affected by Ca²⁺ in the absence of rREG₂⁹K. The reversibility of the Ca²⁺-dependent inhibition of rREG₂⁹K-MCP complexes is illustrated by the data in Fig. 3D; rREG₂⁹K-MCP complexes formed in the presence of 300 μM Ca²⁺ recovered up to 90% of their activity after increasing amounts of EGTA were added. The dose dependence and reversibility of Ca²⁺ inhibition of rREG₂⁹K-MCP complexes were observed using two separate preparations of MCP and three different preparations of recombinant REG. These effects were also observed with the 11 S regulator obtained directly from red blood cells. However, the Ca²⁺-activated protease, calpain, is present in the partially purified red blood regulator fraction, which complicates interpretation of calcium effects on MCP peptidase activity.

Fig. 2. Effect of Mg²⁺ on 45Ca²⁺ binding to test proteins. The test proteins (1 μg) each of bovine serum albumin (BSA), Ub, calmodulin (CaM), rREG₂⁹K, Ub-KEKE, and Ub-KEKE½ were applied to a nitrocellulose membrane and probed with approximately 2 μM 45Ca²⁺ as described under “Experimental Procedures.” The presence of equal amounts of protein on the nitrocellulose filters was confirmed by Ponceau S staining.

2 W. Dubiel, unpublished observation.
This conclusion is reinforced by circular dichroism spectra from the presence of 300 m regions containing KEKE motifs. However, the Ca2+ and the ryanodine receptor (32) bind calcium in regions complexes. MCP (100 ng) was incubated with 100 m Ca2+ binding proteins do not possess either structure; nonameric binding sites. Ruthenium red and45Ca2+ bound45Ca2+ presented above provide strong evidence that calcium binds not precisely localized within the expressed peptides, which presence of 1 mM EGTA (open circles) in the room temperature. The experiments presented above do not address the mechanism by which Ca2+ inhibits rREG29K-MCP peptidase activity. The stimulatory effect of the REG depends on its physical interaction with MCP (17). Glycerol gradient and native gel analysis of rREG29K-MCP mixtures suggest that the complexes dissociate in the presence of Ca2+, but the experiments are not conclusive because of the low affinity of rREG29K for the multicatalytic protease. It is also unclear that the Ca2+ effect is mediated only by rREG29K. Native MCP applied to nitrocellulose binds both 45Ca2+ and ruthenium red,4 and α-subunits of MCP contain KEKE motifs (20). Thus, the rREG29K-MCP interaction could result in conformational changes that activate Ca2+ binding by MCP subunits, and this event might inhibit peptide hydrolysis. Discovering how Ca2+ inhibits rREG29K-MCP peptidase activity will require further experimentation.

In summary, we have demonstrated that the 29-kDa subunit of REG is a calcium-binding protein. We have also provided strong evidence that the KEKE motif present in rREG29K is a Ca2+-binding site. Although the experiments show that Ca2+ reversibly inhibits peptide hydrolysis by rREG29K-MCP complexes, the molecular mechanism has not been discovered. Moreover, the physiological significance of this finding remains an open question. Nonetheless, there is a real possibility that intracellular calcium levels regulate proteolysis by the multicatalytic protease.

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