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Exchange characteristics of calcium ions bound to anthrax protective antigen☆

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Abstract

Protective antigen (PA), the receptor-binding moiety of anthrax toxin, contains two calcium atoms buried within domain 1′ (amino acid residues 168–258). We showed that these ions are stably bound and exchange with free $^{45}Ca^{2+}$ only slowly ($t_{1/2} \sim 4.0$ h). Dissociation is the rate-limiting step. PA_{63}, the heptameric prepore form of PA, showed a slightly higher exchange rate than the monomeric intact protein. Exchange by this form was retarded by binding of the enzymatic moieties of the toxin, but was unaffected by reducing the pH to 5.0, a condition known to trigger conversion of the prepore to the pore form. These results are consistent with the hypothesis that bound Ca^{2+} within PA plays primarily a structural role, maintaining domain 1′ in a conformation that allows PA_{63} to oligomerize and bind the enzymatic moieties of the toxin.

Keywords: Anthrax toxin; Protective antigen; Calcium ion; Exchange rate; Ligand binding

Anthrax toxin consists of three monomeric proteins that assemble after their release from the *Bacillus anthracis* into toxic hetero-oligomeric complexes [1]. Two of them, edema factor (EF) and lethal factor (LF), are enzymes that act on substrates within mammalian cells, and the third, protective antigen (PA), delivers EF and LF to the cytosol. EF is a calmodulin-dependent adenylate cyclase [2], and LF is a zinc-dependent protease that cleaves certain MAP kinase kinases and possibly other cytosolic targets [3–5].

Assembly of the toxic complexes can occur either in solution or at the surface of receptor-bearing mammalian cells. Cell-associated assembly begins when the PA (83 kDa) binds to its receptor in the plasma membrane (ATR) [6] and is proteolytically activated by furin or furin-like protease [7]. Proteolytic removal of a 20 kDa fragment (PA_{20}) from the N-terminus allows the remaining receptor-bound fragment (PA_{63}, 63 kDa) to oligomerize, generating a ring-shaped heptamer [8]. This heptamer, termed the prepore, binds up to three molecules of EF and/or LF competitively and with high affinity [9]. A similar assembly process can occur in solution after tryptic activation of PA, and there is evidence to suggest that such complexes are also generated in the blood of infected animals at late stages of the disease process [10].

The receptor-bound complexes are endocytosed and trafficked to an acidic compartment within the cell [11], where the low pH induces a conformational change in the prepore, converting it to a membrane-spanning pore. This conversion is required for translocation of EF and LF across the membrane to the cytosol [12]. There is good evidence that prepore-to-pore conversion involves formation of a transmembrane 14-strand β-barrel [12,13].

The crystal structure of PA revealed the presence of two calcium atoms buried within domain 1 [14]. These ions are in the C-terminal part of that domain (termed domain 1′), which remains associated with PA_{63} after removal of PA_{20}. Recent evidence that domain 1′ functions in binding the enzymatic ligands, EF and LF, to the PA_{63} heptamer [15] lends credence to the proposal of Petosa et al. [14] that the calcium atoms stabilize this domain in a functional conformation. Here we report experiments to characterize these bound ions. We have

☆ *Abbreviations:* PA, protective antigen; LF, lethal factor; EF, edema factor; LFn, amino-terminal domain of LF.

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measured their rates of exchange with free Ca^{2+} under various conditions and relate our results to the role of these ions in toxin action.

Materials and methods

Protein purification. An expression vector pET22b containing the DNA fragment encoding PA was transformed into *Escherichia coli* (BL21-DE3) (Novagen) and PA was expressed as described previously [12]. The protein was purified to at least 90% homogeneity as judged by SDS–PAGE and stored at −80 °C.

Preparation of heptameric PA_{63} prepore. Nicked PA (nPA) was prepared by incubating PA with trypsin (Sigma) at a ratio of 1000:1 (w:w) at room temperature for 30 min followed by addition of a 10-fold excess of soybean trypsin inhibitor to terminate the reaction. PA_{63} heptamer was prepared from nicked PA by purification on a Mono Q HR5/5 column in 20 mM TAPS, 20 mM PIPES (pH 8.8) with a 0–0.4 M NaCl gradient. For the experiments performed under acid conditions, PA_{63} prepore was incubated in 20 mM MES, 5 mM gluconate, and 140 mM NaCl (pH 5.0) at room temperature for 1 h.

Preparation of apo-PA. PA was partially unfolded at 25 °C for 2 h in 3 M urea and 5 mM EGTA (in buffer H: 20 mM Hepes, 150 mM NaCl, pH 7.4, for PA; or in buffer T: 20 mM TAPS, 20 mM PIPES, and 150 mM NaCl, pH 8.8, for PA_{63}) and then dialyzed against buffer H (PA) or buffer T (PA_{63}) containing 1 mM EGTA at 4 °C.

Determination of calcium content and protein concentration. All buffers were treated with Chelex-100 resin (Bio-Rad) to remove trace metals. Total Ca^{2+} content of PA was determined with a Perkin–Elmer Flame Atomic Absorption Spectrophotometer 2280. Protein concentration was determined spectrophotometrically using a molar extinction coefficient of 78575 $M^{-1}cm^{-1}$ at 280 nm in 6 M guanidine–HCl and 30 mM MOPS (pH 8.0), or by the method of Bradford with bovine serum albumin as a standard.

Preparation of ^{45}Ca-containing PA. PA was incubated with a 25-fold molar excess $^{45}CaCl_2$ (NEN) in buffer H at 37 °C for 24 h. Free calcium ions were removed on a Sephadex G-25 column. The labeling efficiency was 1.9–2.0 $^{45}Ca^{2+}$ per molecule of PA. $^{45}Ca^{2+}$ labeling of PA_{63} heptamer was carried out in buffer T at 37 °C for 8 h before desalting.

Calcium exchange. To determine the rate of $^{45}Ca^{2+}$ incorporation, PA or PA_{63} heptamer (4 μM) was mixed with 100 μM $^{45}CaCl_2$ and incubated at 37 °C for various periods. To measure the rate of $^{45}Ca^{2+}$ dissociation, ^{45}Ca-labeled PA was incubated with $CaCl_2$. LFn was present at 4-fold molar-excess, where indicated. Two 4–20% native gradient gels (BMA) were run in parallel for each set of samples. One was used for quantification of ^{45}Ca-containing PA or PA_{63} heptamer. It was dried and then exposed to a Bio-Rad Molecular Imaging Screen. Quantification of band intensity was performed using Molecular Imager software (Bio-Rad). The other gel was stained with silver or Coomassie brilliant blue, and the intensity of each band was quantified with Molecular Imager Fluo-S software to estimate protein. The amount of $^{45}Ca^{2+}$ at each time point was normalized for protein level.

Intrinsic fluorescence analysis. Intrinsic fluorescence spectra were taken at a protein concentration of 1 μM with excitation wavelength at 280 nm using an ISS K2 spectrofluorimeter at 25 °C.

Results

Quantification of Ca^{2+} in native PA by atomic absorption gave a value of 1.84 ± 0.06 mol Ca^{2+}/mol protein, consistent with 2 Ca^{2+} seen in domain 1 by X-ray crystallography [14]. The Ca^{2+} was not removed by extensive dialysis against 5 mM EGTA alone, but treating the protein with 5 mM EGTA in the presence of 3 M urea, followed by extensive dialysis against 1 mM EGTA, yielded a product devoid of Ca^{2+} (apo-PA), as determined by atomic absorption.

The fluorescence emission λ_{max} of holo-PA, with excitation at 280 nm, was 324 nm, whereas that of apo-PA was 338 nm, close to that of PA in 3 M urea. When excess $CaCl_2$ was added to apo-PA, the emission spectrum was blue-shifted, giving a λ_{max} of 328 nm and suggesting partial reconstitution. Apo-PA migrated more slowly on native PAGE than holo-PA and on a size-exclusion column eluted as a broad asymmetric peak, suggesting heterogeneity or affinity for the chromatographic medium (data not shown). Apo-PA also gave a different trypsin cleavage pattern than holo-PA. Whereas native PA gave two bands on SDS–PAGE, corresponding to PA_{63} and PA_{20}, apo-PA yielded PA_{20} plus two other bands corresponding to fragments of 30 and 31 kDa (Fig. 1).

Despite the fact that Ca^{2+} was not removed from native PA with 5 mM EGTA, incubation of the protein with $^{45}CaCl_2$ at 37 °C (25-fold molar excess, relative to the protein) resulted in slow incorporation of the isotope, as determined by native gel electrophoresis, followed by autoradiography. The time course indicated a monophasic pseudo-first-order reaction. The kinetics fitted well to the equation:

$$Y = Y_{max}(1 - e^{-kt}),$$

where t is the incubation time, k is the incorporation rate constant, Y_{max} is the maximum incorporation yield, and Y is the incorporation percentage at time t. The rate of dissociation of $^{45}Ca^{2+}$ from PA measured in the presence of excess unlabeled $CaCl_2$ corresponded closely to the rate of incorporation of $^{45}Ca^{2+}$ into PA in the presence

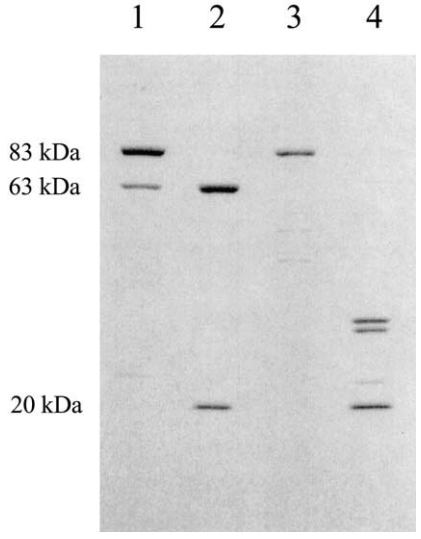

Fig. 1. SDS–PAGE analysis of trypsin-cleavage pattern of PA and apo-PA. PA (lane 1), nPA (lane 2), apo-PA (lane 3), and trypsin-cleaved apo-PA (lane 4). A small fraction of the protein in lane 1 had been cleaved at the furin site by a contaminating protease.

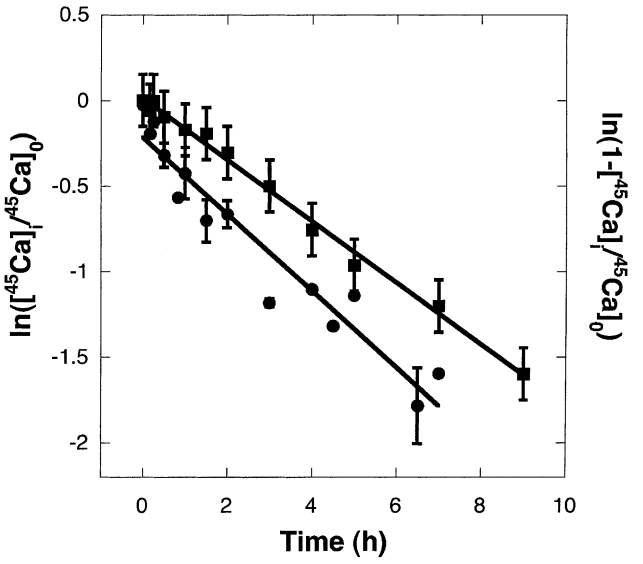

Fig. 2. Time course of Ca^{2+} exchange in PA. PA–Ca^{2+} (●) or PA–$^{45}Ca^{2+}$ (■) of 4 μM was incubated at 37 °C with 25-fold excess $^{45}CaCl_2$ or $CaCl_2$, respectively, for various times. Samples were taken at different time points and run on a 4–20% native gel for quantification.

of excess $^{45}CaCl_2$ (Fig. 2). A rate constant of $0.20\,h^{-1}$ and a half-time of dissociation of ~ 4.0 h were derived from the data (Table 1). Ca^{2+} association to apo-PA was complete within a few minutes, as determined by the change in intrinsic fluorescence. The results are consistent with binding of Ca^{2+} to PA being strong but reversible, and dissociation being the rate-limiting step.

When we incubated purified PA_{63} heptamer at pH 8.8 with a 25-fold molar excess of $^{45}CaCl_2$ (relative to the PA_{63} subunit concentration), the rate of $^{45}Ca^{2+}$ incorporation was about 1.8-fold higher than with PA (Table 1). The rates of association and dissociation of $^{45}Ca^{2+}$ at pH 5.0 were the same as those at pH 8.8 (Fig. 3A).

Evidence from mutagenesis experiments supports the notion that LF and EF bind to the surface of domain 1′ exposed after release of PA_{20}. Thus these ligands might be predicted to stabilize the binding of Ca^{2+} to PA_{63}. As shown in Fig. 3B, binding of LFn, the N-terminal domain of LF, to PA_{63} under conditions of saturation did in fact cause a slight decrease in the rate of $^{45}Ca^{2+}$ incorporation, both at pH 8.8 and pH 5.0.

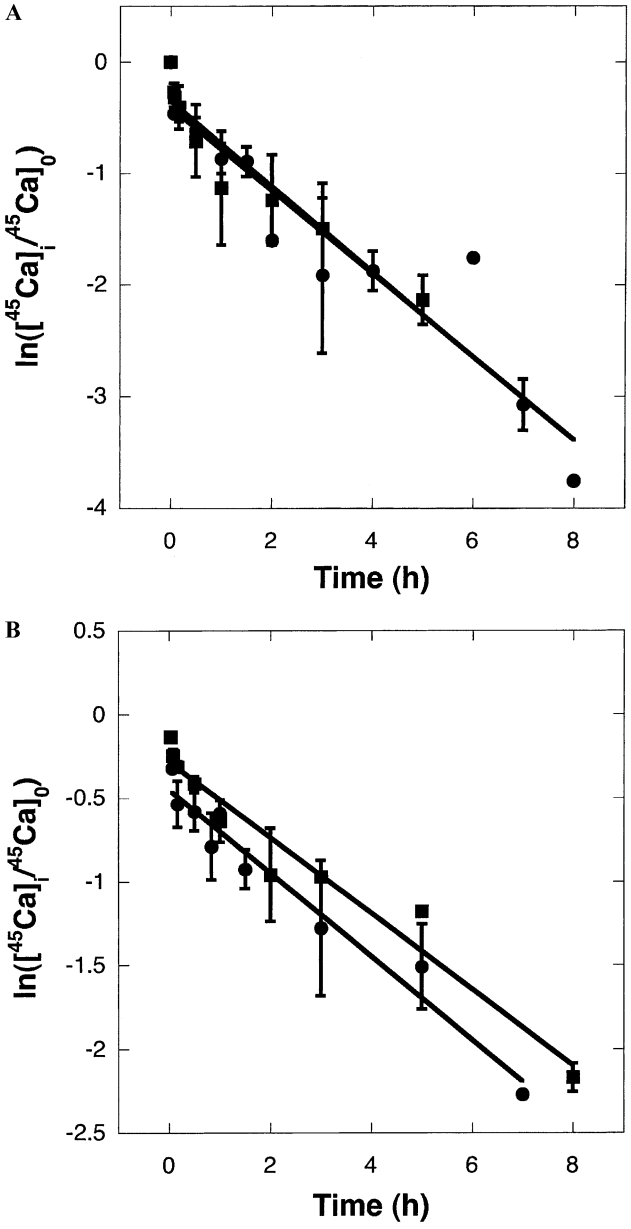

Fig. 3. Time course of Ca^{2+} exchange in PA_{63} heptamer in the absence (A) or presence (B) of LFn. PA_{63}–Ca^{2+} was incubated at 37 °C with 25-fold excess of $^{45}CaCl_2$ at pH 8.8 (●) or pH 5.0 (■) with or without 4-fold excess of LFn. Samples were processed as described in Fig. 3.

Discussion

The results presented are consistent with the notion that the two Ca^{2+} ions within domain 1′ of PA play principally a structural role, maintaining that domain in a functional conformation. These ions are tightly bound in both native PA and the heptameric PA_{63} prepore; exchange with free Ca^{2+} in the medium is slow, with a $t_{1/2}$ of 4 h. No evidence of heterogeneity in behavior of

Table 1
Rate constants of calcium exchange in PA

	PA	PA_{63} heptamer		PA_{63} heptamer + LFn	
		pH 8.8	pH 5.0	pH 8.8	pH 5.0
Rate constant (h^{-1})	0.20 ± 0.03	0.37 ± 0.03	0.38 ± 0.04	0.25 ± 0.05	0.23 ± 0.03

the two ions was detected in our measurements of the kinetics of exchange with free Ca^{2+}. This is consistent with the fact that the two ions occupy a twin site, in which both are coordinated by the side-chain carboxylates of certain residues (D179, D181, and E188) [14]. Hence the two sites are intimately linked, and any molecular perturbation that promotes exchange of one of the ions is likely also to promote exchange of its twin.

The exchange rate measured with the heptameric PA_{63} was slightly elevated, by about twofold, relative to that of the intact protein, perhaps reflecting a change in the mobility of domain 1′ following release of PA_{20} and self-association of PA_{63}. Domain 1′ has been implicated in PA_{63} subunit–subunit interactions, as well as ligand binding [16]. The fact that complexing of the prepore with LFn decreased the exchange rate presumably reflects ligand-dependent stabilization of domain 1′.

Our measurements of the rate of Ca^{2+} exchange from PA_{63} under acidic conditions are relevant to concepts of how EF and LF are translocated across the endosomal membrane. For EF and LF to be translocated to the cytosol their interaction with PA_{63} presumably must be disrupted. Surface plasmon resonance measurements and studies with whole cells show that these enzymatic ligands bind to the PA_{63} heptamer tightly, with K_d values near 1 nM [17]. The acidic conditions that trigger the change in conformation leading to pore formation could conceivably also bring about release of ligands, through a pH-dependent change in the conformation either of domain 1′ of PA_{63} or of the homologous N-terminal domains of EF and LF, or of both. The finding that lowering the pH to 5.0 did not affect the rate of Ca^{2+} exchange within PA_{63} implies that domain 1′ does not undergo significant change in conformation under acidic conditions. This is in agreement with results of surface plasmon resonance and cell-based measurements [17].

Acknowledgments

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