PAD3 autocitrullination and the structure

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Abstract

Peptidylarginine deiminases (PADs) are enzymes that catalyze the Ca^{2+}-dependent conversion of arginine residues in proteins to citrulline residues. Five PAD isozymes have been identified in mammals. Several studies have shown that the active-site pockets of these isozymes are formed when Ca^{2+} ions are properly bound. We previously characterized the structures of PAD3 in six states. Among these, we identified a "non-productive" form of PAD3 in which the active site was disordered even though five Ca^{2+} ions were bound (Funabashi et al., 2021, **Figure 1**). This strange structure was probably obtained as a result of either high Ca^{2+} concentration (~260 mM)-induced denaturation during the crystallization process or high Ca^{2+}-concentration-induced autocitrullination. While autocitrullination has been reported in PAD2 and PAD4 for some time, only a single report on PAD3 has been published recently. In this study, we investigated whether PAD3 catalyzes the autocitrullination reaction and identified autocitrullination sites. In addition to the capacity of PAD3 for autocitrullination, the autocitrullination sites increased depending on the Ca^{2+} concentration and reaction time. These findings suggest that some of the arginine residues in the "nonproductive" form of PAD3 would be autocitrullinated. Furthermore, most of the autocitrullinated sites in PAD3 were located near the substrate-binding site. Given the high Ca^{2+} concentration in the crystallization condition, it is likely that Arg372 was citrullinated in the "nonproductive" PAD3 structure, the structure was slightly altered from the active form by citrulline residues, and probably inhibited Ca^{2+}-ion binding at the proper position. Following Arg372 citrullination, PAD3 enters an inactive form; however, the Arg372-citrullinated PAD3 are considered minor components in autocitrullinated PAD3 (CitPAD3), and CitPAD3 does not significantly decrease the enzyme activity. Autocitrullination of PAD3 could not be confirmed at the low Ca^{2+} concentrations seen *in vivo*. Future experiments using cells and animals are needed to verify the effect of Ca^{2+} on the PAD3.

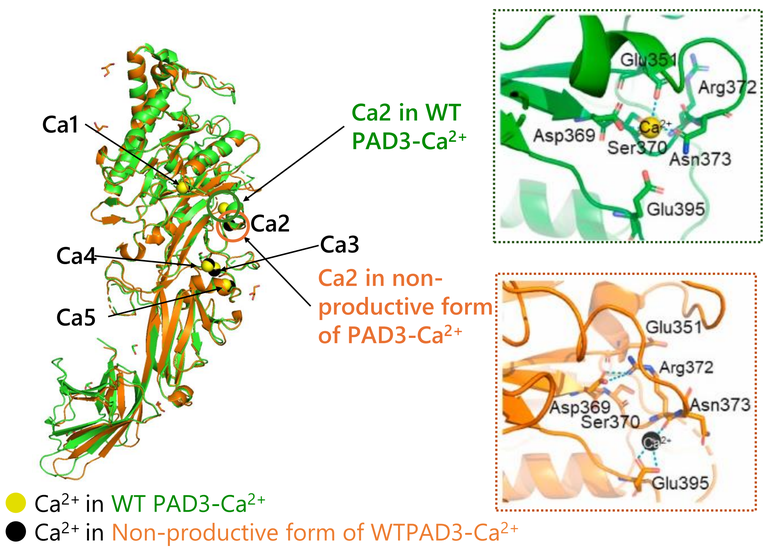

Figure 1. Superimposition of the overall structures of Ca^{2+}-bound WT PAD3 in its active (green-based)- and non-productive (orange-based) forms.

K. Funabashi. *et al.* 2021. *Arch. Biochem. Biophys.* **708.** 108911

Does PAD3 catalyze autocitrullination?

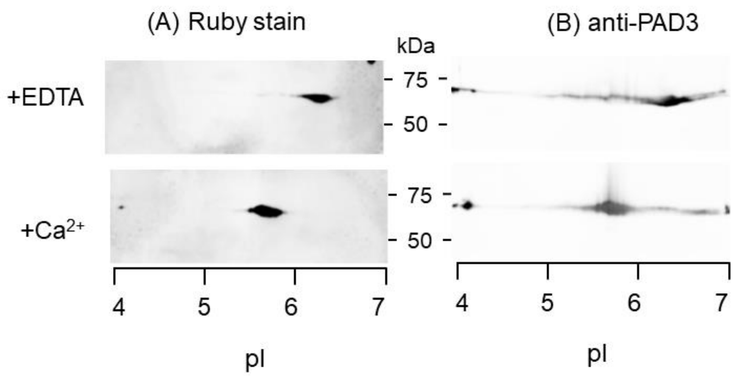

Figure 2. Acidic shift of autocitrullinated PAD3. Aliquots (3 µg each) incubated in the presence of either EDTA (control experiment) or Ca^{2+} and subjected to 2D electrophoresis.
Proteins transferred onto a PVDF membrane were stained with SYPRO ruby for the detection of total protein (A) and subsequently probed with anti-PAD3 antibody (B). WT PAD3 (3 mg/mL) in 20 mM Tris-HCl buffer (pH 7.6) containing 500 mM NaCl, 10% (v/v) glycerol, and 5.0 mM DTT and either 1 mM EDTA (Control experiment) or 10 mM $CaCl_2$ at 37 ºC for 3 h.

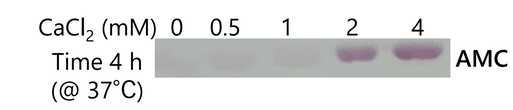

Figure 3. Ca^{2+}-concentration-dependent autocitrullination of PAD3 detected using Western blotting.
The PAD3 concentration used was 0.1 mg/mL.

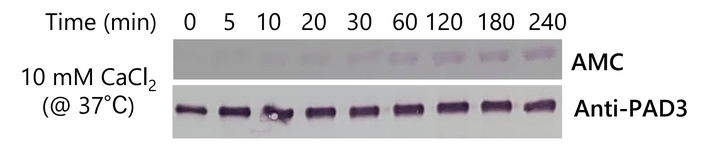

Figure 4. Reaction-time-dependent changes in the citrulline levels at stable PAD3 concentrations detected using Western blotting.
PAD3 concentration: 0.05 mg/mL

- **Yes, PAD3 autocitrullinated 30 to 50% of Arg in the presence of 10 mM $CaCl_2$ in 30 h at 37℃.**
- **PAD3 autocitrullinated Args in a Ca^{2+} concentration- and reaction time-dependent manner.**
- **However, autocitrullination of PAD3 *could not* be confirmed at the low Ca^{2+} concentrations seen *in vivo*.**

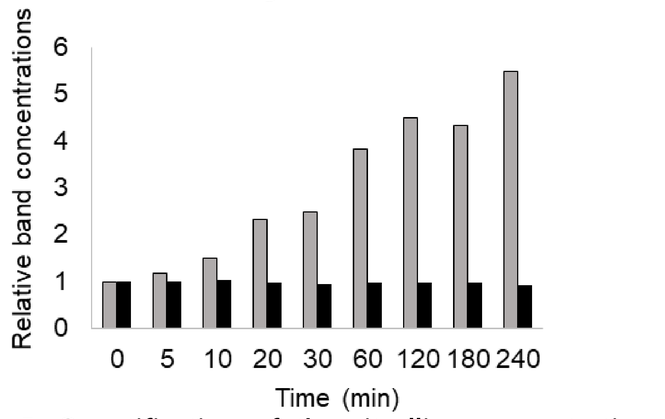

Figure 5. Quantification of the citrulline concentration based on western blotting quantification (Figure 4). The horizontal axis represents time in min. The vertical axis represents the relative value of the band intensity of 0 min as 1. Gray bars and black bars represent the relative band concentrations for AMC and anti-PAD3, respectively.

Which arginine residues are citrullinated?

Table 1. Regions containing arginine or citrulline detected using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

	Experiment 1				Experiment 2						Experiment 3			Experiment 4
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
Arg131	N	✓	✓	✓	N	N	N	N	N	N	N	✓	N	✓
Arg314	✓	N	N	✓	N	N	N	N	N	N	N	N	✗	N
Arg343	✗	N	N	✓	✗	✗	✗	✗	✗	✗	✗	✓	✗	✓
Arg346	✗	N	N	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
Arg372	✗	✗	✗	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
Arg394	✗	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
Arg397	N	✓	✓	N	N	N	N	N	N	N	N	N	N	N
Arg399	N	✓	✓	✓	N	N	N	N	N	N	N	✓	✗	✓
Arg427	N	N	N	N	N	N	N	N	N	N	✓	N	✓	✓
Arg447	N	N	✓	N	N	N	N	N	N	N	N	N	N	N
Arg510	N	✓	✓	✓	N	N	N	N	N	N	N	N	N	N

✓, the identified citrullinated arginine moiety; ✗, the non-citrullinated arginine site identified; N, the arginine site not detected in the experiment. The experiments were conducted four times. The L. O. T. of WT PAD3 samples are different from each other. However, for the third and fourth measurements, the L. O. T. of WT PAD3 samples were identical (see Table 1). Arg314 must be a false positive because it was detected in both WT PAD3 without Ca^{2+} and C646A, the inactive mutant.

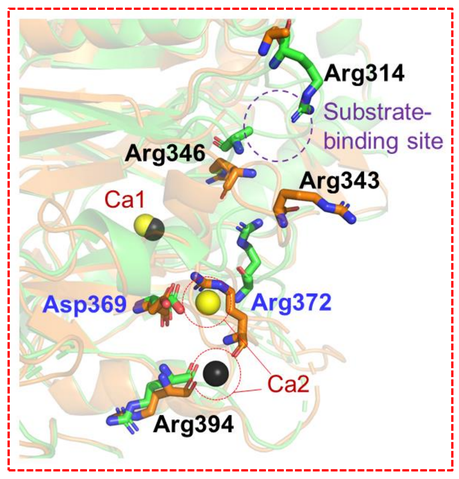

Figure 8. Close-up view of citrullines distribution near the substrate binding site.
The structures of WT PAD3-Ca^{2+} and the "non-productive" WT PAD3-Ca^{2+} are shown in green and orange, respectively. Yellow and black spheres indicate Ca^{2+} in the WT PAD3-Ca^{2+} structure and the "non-productive" WT PAD3-Ca^{2+} structure, respectively. The structure of Arg343 in the WT PAD3-Ca^{2+} was not determined. The purple dotted circle indicates the vicinity with the substrate binding site.

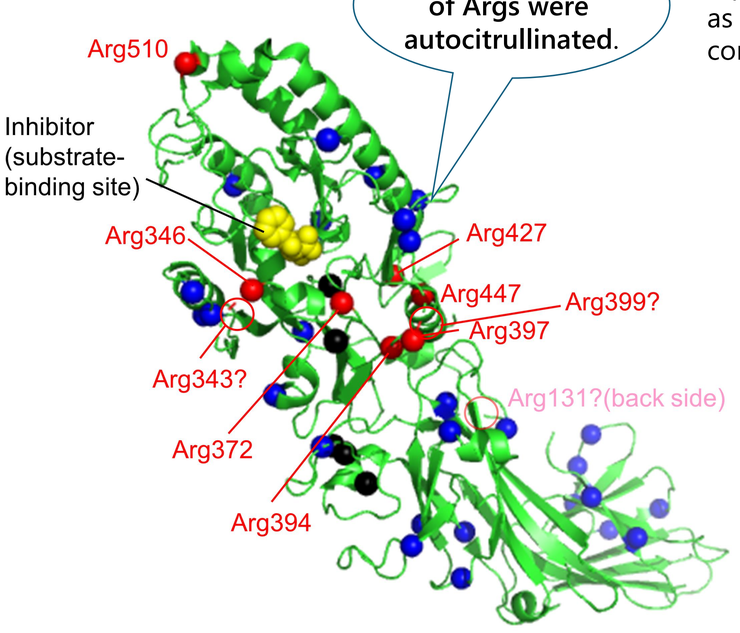

Figure 6. Distribution of arginine and citrullinated arginine residues in PAD3. The structure of WT PAD3-Ca^{2+}-Cl-amidine (Chain A of PDB ID; 7D56) is shown in green. The black spheres indicate Ca^{2+} ions. Blue and red spheres indicate arginine and autocitrullinated arginine residues, respectively. In 7D56, seven of the ten citrullinated arginine residues were visualized. Red circles indicate three autocitrullinated arginine residues (Arg131, Arg343, and Arg399) at putative positions that were not identified following the X-ray crystallographic structure analysis. The inhibitor Cl-amidine is shown in yellow.

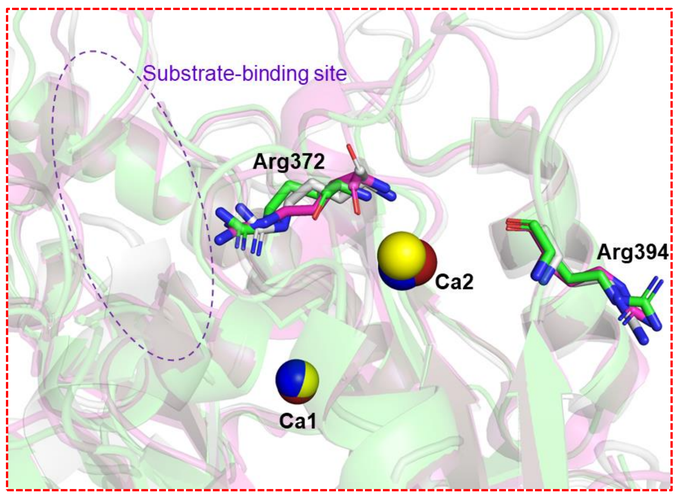

Figure 7. Structures around Arg372, which is citrullinated in PAD2, PAD3, and PAD4, and Arg394, which is citrullinated in PAD3 and PAD4.
The structures of PAD2, PAD3, and PAD4 are all Ca^{2+}-bound forms and colored in gray, green, and magenta, respectively. Only Arg372 and Arg394 (PAD3 numbering) are depicted in stick models. The Ca^{2+} (Ca1 and Ca2) ions bound in the vicinity of the two arginine residues of interest are depicted as red, yellow, and blue spheres in the PAD2, PAD3, and PAD4 structures, respectively. We also examined whether there were common autocitrullinated residues among the different isozymes, and found that Arg372 near Ca2 was commonly autocitrullinated. Furthermore, Arg394 was shown to be autocitrullinated in PAD3 and PAD4, but not in PAD2. These results suggest that Arg372 is one of the residues that is autocitrullinated.

Is citrullinated PAD3 (CitPAD3) active or not?

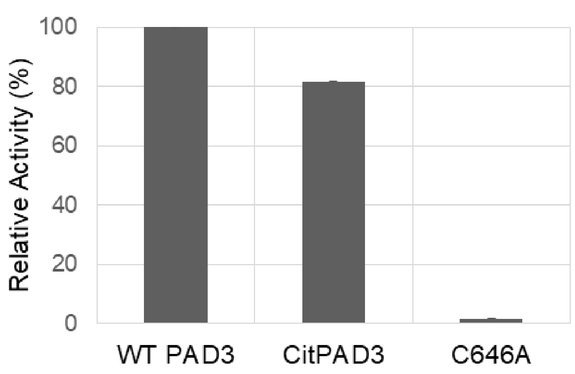

Figure 9. PAD3 activity. From left to right: activity measurements (n = 3) of WT PAD3 (37 °C, 1 h incubation without Ca^{2+}), CitPAD3 (37 °C, 1 h incubation with 10 mM $CaCl_2$), and PAD3 C646A mutant. The vertical axis indicates the activity percentage of WT PAD3 as 100%

→ CitPAD3 is active!

Arg372 citrullinated PAD3 is inactive, but it is different from CitPAD3

- The structure of the 'non-productive' WT PAD3-Ca^{2+} represents the inactive form, and citrullination of Arg372 prevents the formation of the active site.
- The citrullination rate of Arg372 in CitPAD3 is low, with Arg372 being citrullinated under extreme conditions or by chance, but rarely under biochemical conditions.
- Activity measurements were performed under 10 mM $CaCl_2$ and 1 hour incubation conditions, while crystallization conditions for 'non-productive' WT-PAD3-Ca^{2+} contained 260 mM $CaCl_2$ and crystallization took from 5 days to a week.
- In the PAD3 autocitrullination experiments, Arg372 were rarely citrullinated by PAD3, but once Arg372 was citrullinated, the molecule is thought to become almost inactive.
- PAD4 with Arg372 citrullinated lost significant activity, while PAD4 and PAD3 produced in the autocitrullination reaction lost little activity. The citrullination rates of Arg372 and Arg374 in PAD4 are lower than those of other arginine residues (Mondal, S. Nat. Commun., 2021).

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