

## A compact intermediate state of calmodulin in the process of target binding

Solution X-ray scattering is one of the classical techniques to extract information about the structure of target molecules. The technique is as simple as to irradiate X-rays to an aqueous protein solution, in which constituent molecules are randomly oriented. Simple as it is, one may extract various aspects of structural information from the scattering profile, including  $I(0)$  (the forward scattering intensity at a zero angle) and  $R_g$  (radius of gyration).  $I(0)$  is a direct measure of the molecular weight of protein. From  $R_g$ , one can tell the gross shape of the molecule, e.g., the molecule is globular or elongated. An important feature of solution X-ray scattering is that, unlike in protein crystallography, one may perform experiments under physiological conditions, under which proteins can undergo reactions, association and dissociation as would occur in live cells. Here we describe an example in which, by combining this classical technique with intense synchrotron radiation from SPing-8 and state-of-the-art detecting techniques, reaction-associated structural changes of a protein are followed with an unprecedented time resolution.

Calmodulin (CaM) is a low-molecular weight (17 kDa) ubiquitous  $\text{Ca}^{2+}$ -binding protein, found in all eukaryotic cells. CaM binds to various target proteins and is implicated in various  $\text{Ca}^{2+}$ -mediated cellular processes including cell growth, proliferation, motility, and apoptosis. A single CaM molecule consists of two globular domains (called N-lobe and C-lobe), connected by a flexible linker. Each of the globular domains has 2  $\text{Ca}^{2+}$ -binding motifs (EF-hand motifs), so that the entire molecule can bind up to 4  $\text{Ca}^{2+}$  ions. It is known that in the process of  $\text{Ca}^{2+}$ -binding and subsequent binding to target proteins, CaM undergoes a characteristic structural change: When CaM is free of  $\text{Ca}^{2+}$  and its target protein, its linker is in an extended form, so that the entire molecule is dumbbell-shaped. Once  $\text{Ca}^{2+}$  ions are bound, CaM binds to its target protein. In this state the linker is disordered and the entire molecule assumes a more compact structure.

The time course of  $\text{Ca}^{2+}$ -induced structural change of CaM was studied in detail in the high-flux BL40XU beamline. The reaction was started by the light-induced decomposition (photolysis) of caged calcium. A caged substance is an inactive chemical, which is decomposed by intense ultraviolet light and releases a biologically active substance. By decomposing caged calcium by an intense Nd-doped yttrium-aluminum-garnet (YAG) pulse laser (light pulse duration, <10 ns), one may increase the  $\text{Ca}^{2+}$  concentration in less than a millisecond. Instead of a target protein, a small

polypeptide was used (mastoparan, a component of wasp venom). Target proteins are usually larger than CaM, but this peptide is even smaller than CaM, so that it does not mask the structural change of CaM when bound to it.

Irradiation of proteins with X-rays causes radiation damage, and with the extremely intense X-rays from the BL40XU beamline, radiation damage occurs in a matter of milliseconds. To avoid this, the solution containing CaM, mastoparan and caged calcium was put in a thin-walled quartz capillary, and was continually moved along its axis during exposure. By doing so, one can minimize the X-ray dose to each part of the sample. The X-ray scattering was recorded with an ultrafast CMOS video camera at a rate of 2,000 frames/s. At a certain timing of X-ray exposure, the YAG laser was flashed, so that the recorded X-ray movie contained the scattering before flash, and the time course of change of scattering profile after the jump of  $\text{Ca}^{2+}$  concentration.

Figure 1 shows the time course of change of  $R_g$  before and after photolysis, determined in

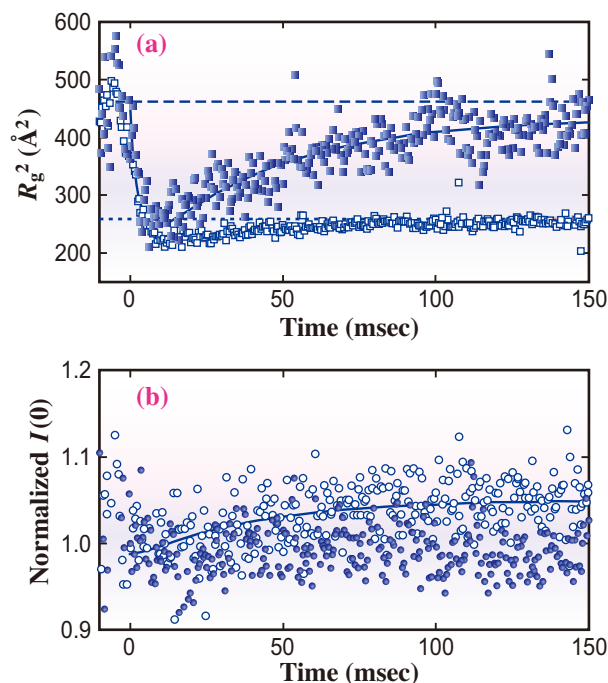


Fig. 1. Results of time-resolved solution X-ray scattering experiments for CaM with and without mastoparan. (a)  $R_g^2$  after photolysis of caged  $\text{Ca}^{2+}$ : (filled square) without mastoparan and (open square) with mastoparan. The dotted and dashed lines show the average values with and without mastoparan, respectively. (b)  $I(0)$  after photolysis of caged  $\text{Ca}^{2+}$ : (filled circle) without mastoparan and (open circle) with mastoparan. The data were normalized by average  $I(0)$  before Ca release.

this way. In the presence of both caged  $\text{Ca}^{2+}$  and mastoparan (Fig. 1(a), open square), the value of  $R_g$  is large ( $\sim 2.0$  nm, meaning that the CaM molecule is in an extended, dumbbell-shaped form) before photolysis. After photolysis, the  $R_g$  is reduced to a low value ( $\sim 1.4$  nm, meaning that the CaM molecule is in a compact form) within 10 ms and stays low. This final form with low  $R_g$  is in agreement with previously published results of static measurement. On the other hand, in the presence of caged  $\text{Ca}^{2+}$  but in the absence of mastoparan (Fig. 1(a), filled square),  $R_g$  is temporarily reduced within 10 ms but gradually returns to a higher value. This indicates that, upon  $\text{Ca}^{2+}$  binding alone, CaM transiently assumes a compact form but becomes extended again, as has been observed in previous studies.

Figure 1(b) shows the time course of change of  $I(0)$ , the measure of molecular weight. In the absence of mastoparan (filled circles), the  $I(0)$  stays low for the entire period of measurement. On the other hand, the  $I(0)$  gradually increases by  $\sim 10\%$  in the presence of mastoparan. This can be explained by slow binding of mastoparan to CaM, because the molecular weight of mastoparan is  $\sim 10\%$  of that of CaM.

Taken together, CaM is quickly transformed to

a compact form after photolysis in the presence of mastoparan, but this is not due to the quick binding of mastoparan to CaM as previously believed, because the mastoparan binding ( $I(0)$  increase) is much slower than the reduction of  $R_g$ . Whether mastoparan is present or not, CaM quickly ( $<10$  ms) assumes a compact form upon  $\text{Ca}^{2+}$  binding alone, and this new compact form has not been reported before. Model calculations of reaction kinetics (Fig. 2) suggest that the new compact form occurs when  $\text{Ca}^{2+}$  binds to the C-lobe alone, because the  $\text{Ca}^{2+}$  binding to the C-lobe is faster than that to the N-lobe. Finally, both lobes become filled with  $\text{Ca}^{2+}$  and the CaM molecule become extended again in the absence of mastoparan, but is stabilized in a compact form in the presence of mastoparan.

CaM is one of the most intensively studied proteins, and the proposal of a new reaction scheme (Fig. 2) for such a protein was made possible by the high time resolution provided by the intense synchrotron radiation and advanced detection techniques. The experimental technique as described here is expected to be useful in studying fast reactions of other proteins, for which conventional techniques like stopped-flow mixing is too slow for the purpose.

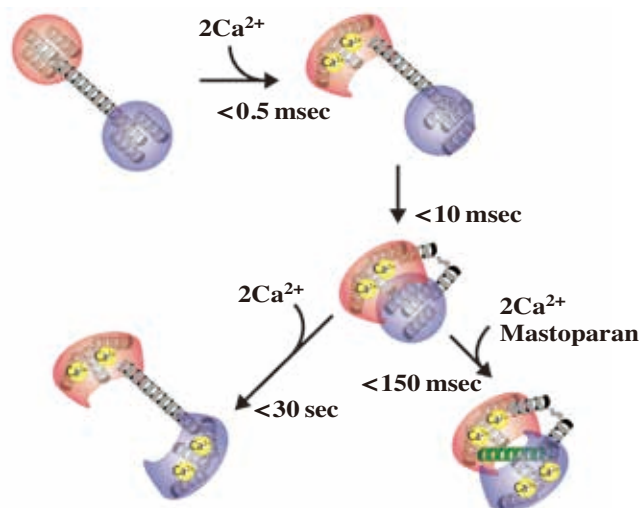


Fig. 2. Schematic illustration of the kinetic reaction of CaM upon  $\text{Ca}^{2+}$  binding. The N- and C-lobes and  $\text{Ca}^{2+}$  ions are colored blue, red, and yellow, respectively. The green helix shows mastoparan. Helices in each domain are colored gray.

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#### References

[1] Y. Yamada, T. Matsuo, H. Iwamoto and N. Yagi: *Biochemistry* **51** (2012) 3963.

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