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Article

In-cell NMR spectroscopy of proteins inside *Xenopus laevis* oocytes

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Abstract

In-cell NMR is an application of solution NMR that enables the investigation of protein conformations inside living cells. We have measured in-cell NMR spectra in oocytes from the African clawed frog *Xenopus laevis*. ^{15}N-labeled ubiquitin, its derivatives and calmodulin were injected into *Xenopus* oocytes and two-dimensional ^{1}H–^{15}N correlation spectra of the proteins were obtained. While the spectrum of wild-type ubiquitin in oocytes had rather fewer cross-peaks compared to its in vitro spectrum, ubiquitin derivatives that are presumably unable to bind to ubiquitin-interacting proteins gave a markedly larger number of cross-peaks. This observation suggests that protein–protein interactions between ubiquitin and ubiquitin-interacting proteins may cause NMR signal broadening, and hence spoil the quality of the in-cell HSQC spectra. In addition, we observed the maturation of ubiquitin precursor derivative in living oocytes using the in-cell NMR technique. This process was partly inhibited by pre-addition of ubiquitin aldehyde, a specific inhibitor for ubiquitin C-terminal hydrolase (UCH). Our work demonstrates the potential usefulness of in-cell NMR with *Xenopus* oocytes for the investigation of protein conformations and functions under intracellular environmental conditions.

Introduction

Information about tertiary structures of proteins at atomic resolution gives us critical insights into their molecular functions. Regardless of the importance of accumulating atomic coordinates from purified proteins, it should be remembered that proteins are not trapped in one particular conformation but rather change their structure dynamically in cells. The types of changes that occur with proteins are fairly broad: simple chemical modification such as phosphorylation, acetylation and methylation; post-translational modification by protein tags such as ubiquitin, SUMO, or NEDD8; formation of complexes with other proteins, nucleic acids, membrane phospholipids or other molecules. These modifications of proteins alter their structural properties, which then trigger further biological events mediated by various downstream effectors. Thus, structural changes of proteins in living cells are closely related to cellular events and functional roles of proteins. So called ''in vivo NMR'' allows the investigation of chemical structures of molecules

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inside living cells and organisms (Graaf, 1998). Until recently, such techniques have been mainly used in studying metabolic profiles of small molecular compounds or drugs. As an extension of this in vivo NMR, Dötsch et al. developed NMR techniques to analyze proteins over-expressed in living bacteria (Serber and Dotsch, 2001a; Serber et al., 2001b, 2001c, 2004, 2005). They measured two-dimensional HSQC spectra of the N-terminal domain of the bacterial mercury-detoxification protein MerA which was uniformly labeled with ^{15}N in living *Escherichia coli* cells. In these experiments a biologically relevant difference was detected between spectra acquired from the cells and an in vitro sample. They also measured the two-dimensional HSQC spectrum of calmodulin selectively labeled with ^{15}N on all lysine residues in the cells, through which they demonstrated that calmodulin exists mainly in the Ca^{2+} free form in the cells. Dötsch et al. termed their approach "in-cell NMR" to distinguish it from conventional small molecular "in vivo NMR". An in-cell NMR approach has also been employed in the study of the intracellular structure of "intrinsically disordered proteins" (Dedmon et al., 2002) and in observing molecular interactions between CheY and small molecular compounds (Hubbard et al., 2003), and between ubiquitin and its interacting proteins (Burz et al., 2006) in living *Escherichia coli* cells. In-cell NMR was further developed into a series of three-dimensional triple resonance experiments by Reardon *et al.* (Reardon and Spicer, 2005), in which the backbone assignment of GB1 protein over-expressed in living *Escherichia coli* cells cultured in ^{13}C, ^{15}N-enriched medium was obtained. They utilized the projection-reconstruction method to minimize the NMR measurement time. Recently, $\{^1H\}$-^{15}N heteronuclear NOE experiments were also performed on apocytochrome b5 in living *Escherichia coli* cells, providing quantitative information on backbone dynamic properties of the protein under intracellular environmental conditions (Bryant et al., 2005). The number of publications describing in-cell NMR is increasing gradually. In this paper, we present in-cell NMR, in which living cells derived from a higher eukaryotic organism, *Xenopus laevis*, were employed. The *Xenopus* oocyte is known as a unique "protein expressing vessel", and thus widely used in functional assays of membrane proteins. In these experiments mRNA encoding a membrane protein can be directly delivered into the oocytes via microinjection techniques. The injected mRNAs are then autonomously translated into proteins in the oocyte. We have employed a similar microinjection technique to deliver ^{15}N-labeled protein into oocytes with the aim of observing NMR signals of the labeled proteins in living oocytes. By using this method, we have investigated proteins under intracellular environmental conditions.

Materials and methods

Protein expression and purification

^{15}N-labeled ubiquitin and its derivatives were expressed and purified as described previously (Tenno et al., 2004). Calmodulin was expressed and purified as described previously with minor modifications (Hayashi et al., 1998). Purified proteins were dialyzed against the injection buffer (7.5 mM $MgCl_2$, 88 mM NaCl, 20 mM HEPES (pH 7.5), 2 mM 2-mercaptoethanol, 5% glycerol (v/v)), prior to microinjection.

Preparation of Xenopus laevis oocytes

Adult females of *Xenopus laevis* were purchased from Kato S-kagaku (Chiba, Japan). Portions of ovary were surgically isolated from mature females by conventional methods (Sive et al., 2000). Stage V-VI oocytes were chemically defolliculated (Dumont, 1972) by 2 h of digestion with 2 mg/ml collagenase (Sigma) in Ca^{2+}-free MBS (88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH7.6)), and then washed 3 times with Ca^{2+}-containing MBS (88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM HEPES (pH7.6)). Healthy oocytes were selected under a dissecting microscope based on morphology and pigmentation. These oocytes were stored in Ca^{2+}-containing MBS at 18 °C, and were used within 2 days. Note that *Xenopus* oocytes can remain healthy for several days under controlled conditions (Sive et al., 2000).

Injection of labeled proteins

Microinjection was performed as described with slight modifications (Sive et al., 2000). Each

oocyte was injected with approximately 20 nl of typically 5 mM ^{15}N-labeled proteins via a Narishige IM-300 microinjector (Narishige Co. Ltd., Tokyo, Japan). For the ubiquitin aldehyde treatment, each oocyte was pre-injected with approximately 20 nl of 2.5 ng/nl ubiquitin aldehyde or water (control), 30 min prior to injection of the labeled proteins.

NMR experiments

All NMR data were acquired at 18 °C on a Bruker DRX-500 spectrometer equipped with a 5 mm TCI CryoProbe. All ^{1}H–^{15}N HSQC spectra were acquired at a ^{1}H frequency of 500.032 MHz with a ^{1}H spectral width of 8012.821 Hz and a ^{15}N spectral width of 1621.534 Hz. The matrix size was 1024* × 64* with acquisition times of 39.5 (t_1) and 127.9 ms (t_2). Typically 64 scans per complex t_1 increments were accumulated with a measurement time of approximately 2 h. Data processing and analysis were performed using NMRPipe (Delaglio et al., 1995) and Sparky (Goddard and Kneller, 1999).

Electrophoresis analysis

SDS-polyacrylamide electrophoresis was performed with PANTERA^{TM} gradient gels (gradient 15–25%) (B-Bridge International, Inc.) as described in the manufacturer's instructions. Gels were stained with Coomassie Brilliant Blue. Loaded samples were prepared as follows. Figure 3b, lane 7–9; medium buffer was taken from the NMR sample tube after each run of NMR experiments and subjected to analysis. Lane 10, 11; a group of oocytes were disrupted by pipetting and heated at 85 °C for 5 min. The supernatants were collected by centrifugation for isolation of ubiquitin derivative, and subjected to analysis. Note that all the oocytes subjected to the SDS-polyacrylamide electrophoresis analysis were derived from a single frog to avoid differences due to variations in individual (Figure 3b).

Results

In order to obtain NMR spectra of uniformely ^{15}N-labeled proteins in the intracellular environment, *Xenopus laevis* oocytes were microinjected with labeled proteins. An overview of the experiment is shown in Figure 1. About 220 *Xenopus* oocytes injected with labeled proteins were collected and gently settled in an NMR sample tube. All NMR experiments were carried out at 18 °C as this is the optimal temperature for the maintenance of the viability of oocytes. The measurements to obtain a ^{1}H–^{15}N HSQC spectrum typically took about 2 h. The state of the oocytes

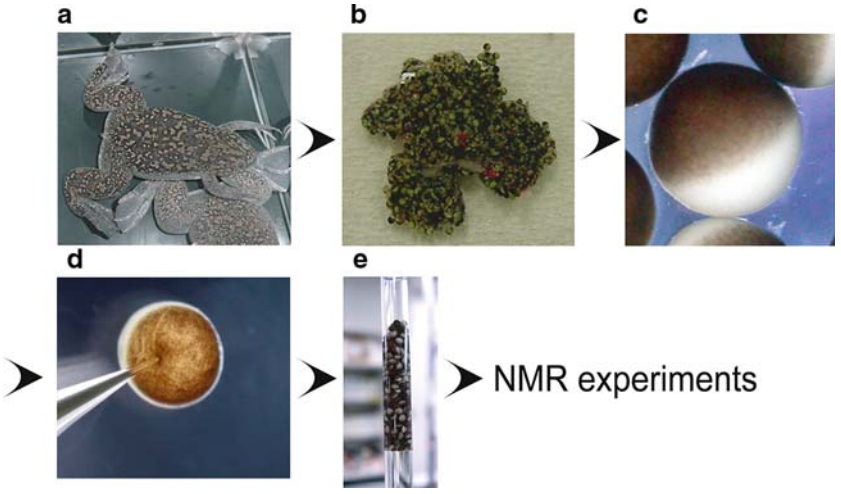

Figure 1. Overview of the experimental procedure. Photographs of an adult female of *Xenopus laevis* (a), an excised ovary (b), a healthy oocyte with a diameter of approximately 1 mm (c), an oocyte under microinjection (d), an NMR sample containing about 220 oocytes in a 5 mm NMR sample tube (e).

used for NMR experiments was checked by microscope observation. A healthy oocyte displays a sharp border between vegetal and animal poles. Although aeration in the NMR tube is not optimal, the oocytes looked healthy after acquisition of at least two ^{1}H–^{15}N HSQC spectra on gross examination (in total about 4.5 h).

In-cell NMR of ubiquitin and its mutants

Wild-type ubiquitin

We chose ubiquitin as the first sample for this study, because it has been well characterized by solution NMR, and plays an important role in cellular events, such as protein degradation via the ubiquitin-proteasome system. Typically 20 nl of 5 mM ^{15}N-ubiquitin solution was injected into each oocyte, giving a final concentration within the oocytes of 0.1 mM (the volume of the oocyte is estimated to be 1 μl) at most. The ^{1}H–^{15}N HSQC spectrum of the ^{15}N-labeled ubiquitin inside the oocytes gave only a few cross-peaks (Figure 2a). This is in striking contrast to the ^{1}H–^{15}N HSQC spectrum of ubiquitin in vitro at the same concentration, which displays well resolved strong cross-peaks attributed to $>95\%$ of the expected main-chain amide ^{1}H and ^{15}N resonances (Figure 4c).

Perturbation of protein–protein interactions improved the quality of in-cell HSQC spectra

We hypothesized that the weakness of the signals observed for ubiquitin in the oocytes was due to extensive protein–protein interactions between ubiquitin and interacting proteins present in the oocytes. To test this hypothesis, experiments were performed to ascertain whether cross-peak intensities of ^{1}H–^{15}N HSQC spectra increased when the interaction surface of ubiquitin was perturbed. To achieve this we focused on three hydrophobic residues, Leu8, Ile44 and Val70, as a number of structural and mutational studies indicate that ubiquitin-interacting down stream proteins commonly recognize the hydrophobic patch formed by these residues (Hicke et al., 2005). We made four ubiquitin mutants by introducing alanine at these positions. These mutants were used in in-cell ^{1}H–^{15}N HSQC measurements using *Xenopus* oocytes. The spectra from these experiments are shown in Figure 2b–e. As hypothesized, recovery of signal intensities was observed for all the mutants. In particular, a dramatic improvement was observed with the mutant in which L8, I44 and V70 had all been replaced with alanine ((L8A, I44A, V70A)-D77 ubiquitin). Most of isolated cross-peaks detected in the in-cell spectra of the mutant proteins are observed at nearly the same positions as those seen in the in vitro spectra (Figure 2e and f). Note that the extra aspartate residue at the C-terminus (D77) and replacement of K48 by Cys in some of these mutant proteins are unlikely to affect protein–protein interactions mediated by the hydrophobic surface of the β-sheet, as these positions are distant from the surface. I44A ubiquitin is

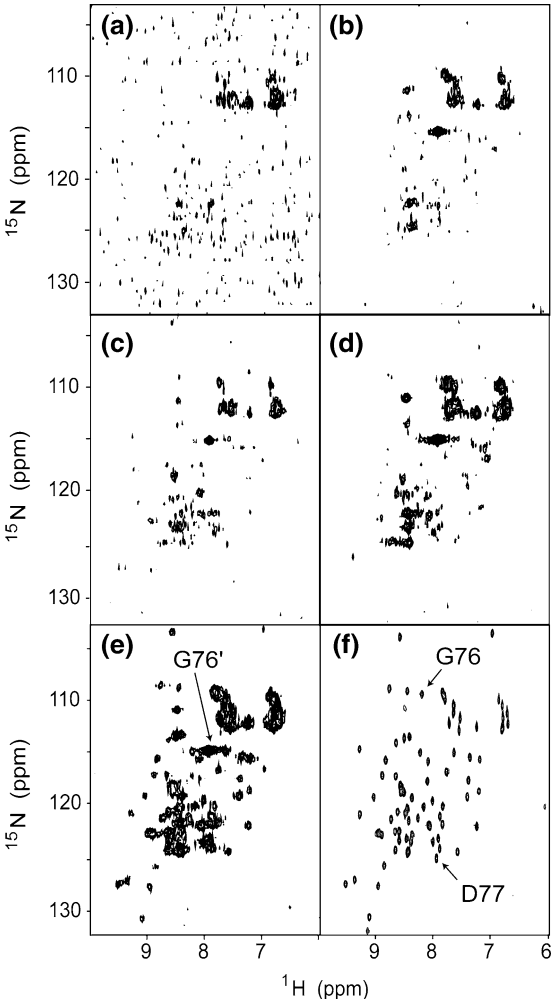

Figure 2. The ^{1}H–^{15}N HSQC spectra of *Xenopus laevis* oocytes injected with wild-type (a), (L8A, K48C)-D77 (b), I44A (c), (K48C, V70A)-D77 (d), (L8A, I44A, V70A)-D77 (e) ubiquitin. The in vitro ^{1}H–^{15}N HSQC spectrum of 10 μM (L8A, I44A, V70A)-D77 ubiquitin in the injection buffer(f). Cross-peaks of G76, D77 and G76′ (the C-terminus G76 after the cleavage of the peptide bond between G76 and D77) are indicated (e, f).

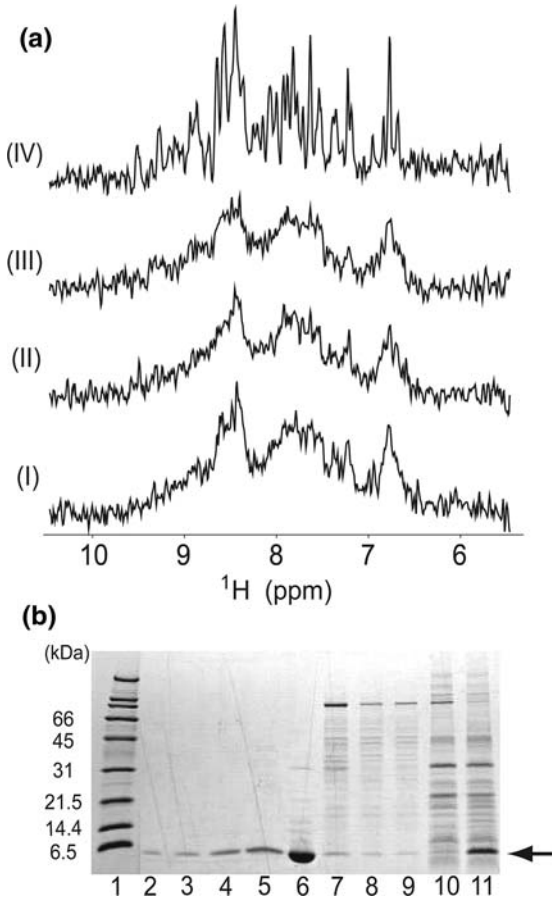

Figure 3. The results of leak test. In panel (a), one-dimensional ^{1}H projections of the ^{1}H–^{15}N HSQC spectra of *Xenopus* oocytes injected with ^{15}N-labeled (L8A, I44A, V70A)-D77 ubiquitin measured before the wash (I), after the first wash (II), and after the second wash (III) are shown. Only a small signal reduction was observed as a result of each wash step, indicating the leakage of the injected proteins were relatively small. One-dimensional ^{1}H projection of the in vitro ^{1}H–^{15}N HSQC spectrum of 20 μM ^{15}N-labeled (L8A, I44A, V70A)-D77 ubiquitin is also shown for comparison (IV). NMR experimental parameters are the same in (I)–(IV). Panel (b) shows SDS-polyacrylamide gel analysis (15–25%) of samples taken from the NMR samples and cell extracts prepared from the oocytes. Lane 1, molecular weight marker; 2–6, ubiquitin control, 1 μl of 1, 2, 5, 10, and 100 μM purified ubiquitin solution was loaded in each lane as references; 7–9, 1 μl of culture medium taken from NMR samples in panel (a), (I), (II) and (III), respectively; 10–11, approximately 1 μl of cell extract of oocytes either not injected or injected with labeled ubiquitin derivative. The arrow marks the location of the ubiquitin derivative band.

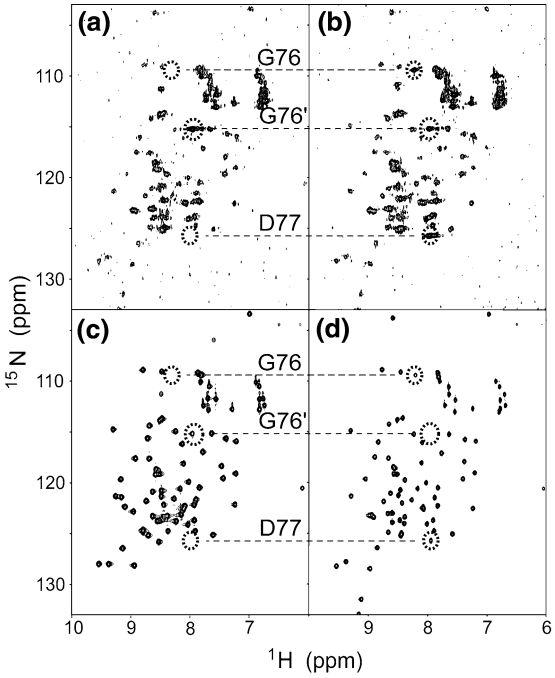

Figure 4. UCH activity in *Xenopus* oocytes monitored by in-cell NMR. The ^{1}H–^{15}N HSQC spectra of oocytes injected with ^{15}N-labeled (L8A, I44A, V70A)-D77 ubiquitin with pre-injection of water (a), and ubiquitin aldehyde (b). The cross-peaks of G76 and D77 are observed at their original positions only with pre-injection of ubiquitin aldehyde. In the spectrum shown in panel (b), the cross-peak attributed to C-terminal G76 is also observed (denoted as G76′). This is presumably the consequence of residual UCH activity. The in vitro ^{1}H–^{15}N HSQC spectra of the wild-type (c) and (L8A, I44A, V70A)-D77 ubiquitin (d) are shown. The cross-peaks attributed to G76, D77 and G76′ are indicated.

derived from yeast, while the others are from human. Although three amino acids are different between these two orthologues, these residues are distant from the hydrophobic surface. Thus, this difference is also unlikely to affect protein–protein interactions with down stream proteins.

In order to show this, in-cell NMR spectra of the following five derivatives from human ubiquitin, wild-type-D77, L8A-D77, I44A-D77, V70A-D77, and (L8A, I44A,V70A)-D77, were measured (Supplementary Figure S1). The signal intensities of spectra of the single amino acid-substituted derivatives (L8A-D77, I44A-D77, V70A-D77) are much smaller than that of the triple amino acid-substituted derivative. The signal intensities in the spectra of wild-type-D77 ubiquitin are significantly smaller than those of all the other derivatives. These features were seen in the spectra shown in Figure 2.

It is noteworthy that there is a possibility of seasonal variation in the quality of in-cell NMR spectra. Out of nine independent in-cell NMR

experiments with (L8A,I44A,V70A)-D77 ubiquitin performed during September to November, seven provided good spectra such as those shown in Figures 2(e) and 4(a), while the remaining two gave reasonable spectra, but the signal intensities are smaller than those seen in the former seven spectra. On the other hand, two independent experiments performed during August, gave much poorer spectra. We assume that this might be, at least partly, due to the seasonal variation of oocytes quality. It is well known that oocytes quality is low during the summer months (Partington et al., 1984; Hilgemann and Lu, 1998; Sive et al. 2000).

Assessment of protein leakage from the oocytes

For in-cell NMR measurements, it is possible that a small amount of injected protein leaks out from the oocytes to the culture medium within the NMR sample tube and thus contributes signals. Since this leakage would seriously affect the interpretation of the NMR spectra, it is important to ascertain whether such leakage does not make a significant contribution to the NMR spectra. In order to minimize the contributions of leaked protein molecules to the signals in the spectra, we carefully chose healthy oocytes which were injected with labeled proteins, and gently but extensively washed them with fresh media (at least 3 times) prior to putting them into the NMR sample tube. To check if the proteins leaked out from the oocytes during NMR measurements, we measured a series of in-cell ^{1}H–^{15}N HSQC spectra of oocytes injected with ^{15}N-labeled (L8A, I44A, V70A)-D77 ubiquitin. Between the measurements, the oocytes were taken out from the NMR tube, carefully washed with fresh media, and then inserted into the NMR sample tube for measurement. The analysis of one-dimensional ^{1}H projections of these HSQC spectra indicated that reduction in peak intensities was approximately 13% after each wash step, thus suggesting that the observed signals are mainly from ^{15}N-labeled proteins within the oocytes (Figure 3a). We also analyzed extracellular proteins by SDS-polyacrylamide gel electrophoresis of the medium buffer taken from the NMR tube after each run of NMR measurements (Figure 3b). Based on these results, we estimate that the concentration of the ubiquitin mutant in the medium buffer was no more than 1 μM. Furthermore, comparison of the in-cell spectra with that of the ubiquitin mutant at 20 μM acquired in vitro with the same acquisition parameters (Figure 3a, IV) allowed us to estimate that the concentration of protein in the cells that gave rise to the in-cell spectrum was approximately 10–20 μM (or even more if line broadening effect is taken into account). This observation is well consistent with the results of SDS-polyacrylamide gel electrophoresis analysis of the oocyte cell extracts used in an in-cell NMR experiment (lane 10 and 11 of Figure 3b). From this analysis we estimated the intracellular concentration of the ubiquitin mutant to be >10 μM. These results allow us to be confident that the leakage of the injected proteins makes negligibly contribution to the spectra.

Observation of the processing of ubiquitin precursor by UCH in oocytes

(L8A, I44A, V70A)-D77 ubiquitin, which gave the most intense signals in the in-cell NMR spectra, contains an additional aspartic acid (D77) at its C-terminus compared to mature ubiquitin which terminates at Gly76. This ubiquitin mutant mimics the ubiquitin precursor, and thus acts as a substrate for ubiquitin C-terminal hydrolase (UCH), which catalyzes hydrolytic cleavage of the peptide bond between G76 and D77 of ubiquitin precursor, generating mature ubiquitin (Johnston et al., 1999). Comparison of the in-cell ^{1}H–^{15}N HSQC spectrum of oocytes injected with ^{15}N-labelled (L8A, I44A, V70A)-D77 ubiquitin with its *in vitro* spectrum shows that the cross-peaks attributed to the amide groups of G76 and D77 are missing in the in-cell spectrum (Figure 2e, f). Instead, one intense cross-peak has emerged at the position which corresponds to G76 of wild-type ubiquitin (Figure 2e, 4c, d). Other than these features, the positions of the cross-peaks were nearly identical in in vitro and in-cell HSQC spectra. These observations strongly suggest that the peptide bond between G76 and D77 of (L8A, I44A, V70A)-D77 ubiquitin is cleaved in the oocytes.

We hypothesized that the cleavage of the G76–D77 peptide bond of (L8A, I44A, V70A)-D77 ubiquitin was performed by endogenous UCH. To confirm this we injected the oocytes with ubiquitin aldehyde, which is a specific inhibitor of UCH activity (Johnston et al., 1999), prior to injection with ^{15}N-labeled (L8A, I44A, V70A)-D77 ubiquitin. An in-cell HSQC spectrum of these oocytes is shown in Figure 4b. In the spectra, the cross-peaks attributable to G76 and D77 were

observed, and that of the C-terminal G76 (denoted as G76′) was smaller than that seen in a spectrum from control oocytes pre-injected with water (Figure 4a), where the cross-peaks of G76 and D77 were not observed. Thus, pre-injection of ubiquitin aldehyde suppressed the disappearance of the cross-peaks of G76 and D77, indicating inhibition of the cleavage of the peptide bond between G76 and D77 of (L8A, I44A, V70A)-D77 ubiquitin.

Figure 5a shows a region of in-cell HSQC spectra of oocytes pre-injected with ubiquitin aldehyde acquired at various times after injection of ^{15}N-labelled (L8A, I44A, V70A)-D77 ubiquitin. Note that the intensity of the cross-peak of D77 gradually decreases, reflecting the presence of residual UCH activity in the oocytes after partial inhibition by ubiquitin aldehyde.

In-cell NMR of Calmodulin

Figure 6a shows an in-cell ^{1}H–^{15}N HSQC spectrum from *Xenopus* oocytes injected with ^{15}N-labeled calmodulin dissolved in a buffer free from Ca^{2+} ions. The spectrum displays a similar pattern to that of apo-calmodulin, although the cross-peaks in the in-cell NMR are broader than those in the in vitro spectrum (Figure 6c). The in-cell spectrum is very different to that of Ca^{2+}-bound calmodulin. These observations indicate that the majority of the injected calmodulin remains in the Ca^{2+}-free form in the oocytes. When an excess of Ca^{2+} ions was co-injected with the labeled calmodulin, the intensities of cross-peaks in the in-cell ^{1}H–^{15}N HSQC spectrum decreased dramatically and the pattern of cross-peaks no longer shows similarities to that of Ca^{2+}-free calmodulin (Figure 6b). Note that the final concentration of Ca^{2+} ions within the oocytes was 1.6 mM (the volume of the oocyte is estimated to be 1 μl) in this experiment. The reduction in signal intensities is clearly demonstrated by comparing one-dimensional ^{1}H projections of these ^{1}H–^{15}N HSQC spectra (top of Figure 6a and b). Although many of the cross-peaks could not be identified because of low signal-to-noise ratio and spectral overlap, more than 10 cross-peaks were found at positions characteristic of Ca^{2+}-bound calmodulin (Figure 6d), suggesting that the injected calmodulin adopted the Ca^{2+}-bound form in the oocytes when co-injected with an excess of Ca^{2+} ions.

Discussion

The ^{1}H–^{15}N HSQC spectrum of oocytes injected with wild-type ubiquitin gave markedly weak

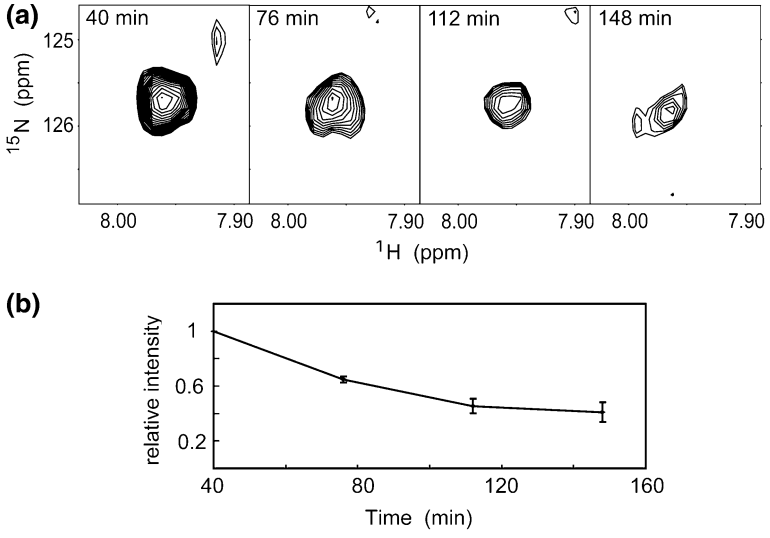

Figure 5. The cross-peak of D77 observed in the ^{1}H–^{15}N HSQC spectra of (L8A, I44A, V70A)-D77 ubiquitin in oocytes with pre-injection of ubiquitin aldehyde, measured at various times after injection of the proteins (a). Acquisition of each spectrum was started at 40, 76, 112 and 148 min after injection. The matrix size was 1024* × 32* with acquisition times of 19.7 (t_1) and 127.9 ms (t_2). About 32 scans per complex t_1 increments were accumulated with a measurement time of approximately 36 min. The contours are plotted with the same threshold levels. The relative peak heights of cross-peaks attributed to D77 are plotted as a function of time. The experiment was replicated twice and the results were shown (b).

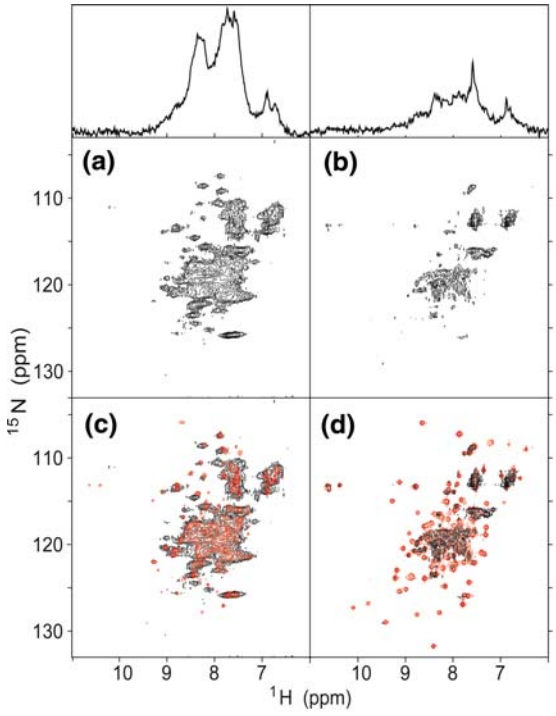

Figure 6. The ^{1}H–^{15}N HSQC spectra of *Xenopus* oocytes injected with ^{15}N-labeled calmodulin. Calmodulin was injected with either 0 mM (a) or 80 mM (b) Ca^{2+} ions. One-dimensional projections taken along the acquisition dimensions are shown on top of both spectra. In vitro ^{1}H–^{15}N HSQC spectra of apo-calmodulin and Ca^{2+} bond calmodulin are overlaid on the corresponding in-cell spectra (c, d). Red – in vitro; black – in-cell spectra.

cross-peaks. One possible cause for these weak signal intensities could be the larger viscosity of the cytosol. However, the viscosity of cytosol has been estimated to be approximately only two fold higher than pure water, based on the measurements of diffusion coefficients by MRI (Sehy et al., 2002). This increase in solvent viscosity is estimated to increase the rotational correlation time of ubiquitin approximately two-fold, and thus have only a moderate effect on the signal intensities of the HSQC spectrum of ubiquitin. We and Fushman *et al*. have reported in vitro ^{1}H–^{15}N HSQC measurements of tetra-ubiquitin chains linked via K48 or K63 (Tenno et al., 2004; Ranjani et al., 2005). Although these ubiquitin chains seem to have more than a 2-fold increase in rotational correlation time compared to a single ubiquitin molecule, they give sharp and intense cross-peaks. In addition, we could obtain much more intense cross-peaks in in-cell HSQC spectra acquired from ^{15}N-labeled apo-calmodulin, whose molecular weight is about twice as large as that of ubiquitin. Based on these facts, the high viscosity of the cytosol is unlikely to be the major reason for the weak signal intensities observed in the in-cell NMR spectrum of wild-type ubiquitin.

Based on the comparisons of ^{1}H–^{15}N HSQC spectra of oocytes injected with wild-type and mutants, we have reasoned that the weak signal intensities obtained from wild-type protein is due to extensive protein–protein interactions with endogenous proteins of oocytes. Ubiquitin plays many roles in regulating cellular processes. In addition to the well-known function of ubiquitin in protein degradation, ubiquitin is involved in a variety of cellular functions, such as endocytosis, transcription and DNA repair. These functions are mediated by a large number of downstream ubiquitin-interacting proteins harboring one or more of ubiquitin binding domains, such as UIM, UBA, CUE, UEV, NZF, and GAT (Hicke et al., 2005). Therefore, it is likely that ^{15}N-labeled ubiquitin injected into oocytes forms stable or transient complexes with these ubiquitin-interacting proteins in the cells, which then causes the large reduction in signal intensities observed in the in-cell NMR spectrum by reason of large molecular weight and/or inhomogeneity of the ubiquitin containing complexes.

Structural and mutational studies have revealed that the hydrophobic patch on the β-sheet of ubiquitin, centered at I44, serves as an interface with most of those ubiquitin-interacting proteins (Hicke et al., 2005; Ohno et al., 2005). We disrupted the hydrophobic patch by substitution of the patch-forming residues with alanine with the aim of reducing protein–protein interactions of ubiquitin within the cell. As expected, HSQC spectra of oocytes injected with the ubiquitin mutants gave more intense signals than observed with wild-type ubiquitin. In particular, simultaneous replacement of L8, I44 and V70 with alanine, which largely disrupts the patch, dramatically increased the signal intensities in the in-cell HSQC spectra. These results are consistent with the assumption by Serber *et al*. that proteins with few interacting partners provide better in-cell NMR spectra (Serber et al., 2001c).

It is noteworthy that we could observe hydrolytic activity of UCH on a ubiquitin derivative in oocytes. To our knowledge, this is the first report of in-cell NMR of proteins enabling real-time

observation of an enzymatic reaction inside cells. This result demonstrates that in-cell NMR can be a tool that provides functional and structural information about various biochemical reactions mediated by proteins within living cells.

The ^{1}H–^{15}N HSQC spectra of oocytes injected with ^{15}N-labeled calmodulin dissolved in Ca^{2+}-free buffer displayed the characteristic pattern of the Ca^{2+}-free form. The Ca^{2+} concentration in the cytosol is known to be of the order of 0.1 μM, whilst the final concentration of the injected ^{15}N-calmodulin in the oocytes is estimated to be of the order of tens of micro molar. Therefore, it is reasonable to observe calmodulin in Ca^{2+}-free form, because the intracellular Ca^{2+} concentration is not high enough to convert the ^{15}N-calmodulin from the Ca^{2+}-free to the Ca^{2+}-bound form. When an excess of Ca^{2+} ions was co-injected, the in-cell spectrum of calmodulin partly gave cross-peaks characteristic of those of the Ca^{2+}-bound form. Interestingly, co-injection with Ca^{2+} ions also caused a marked decrease in intensities of the NMR signals of calmodulin in the in-cell NMR spectrum. This observation is reminiscent of the in-cell NMR experiment with wild-type ubiquitin, in which we reasoned that the low signal intensities were due to extensive protein–protein interactions. Upon Ca^{2+} binding, calmodulin is induced to bind various downstream effector proteins (Zhang and Yuan, 1998). Hence, we speculate that the signal reduction observed for calmodulin upon Ca^{2+} co-injection is attributable to at least partly interactions between Ca^{2+}-bound calmodulin and downstream effector proteins. However, we cannot rule out the possibility that the injection of non-physiological concentration of Ca^{2+} severely perturb the intracellular environment, leading non-specific degradation or aggregation of calmodulin.

In summary, we have observed properties of proteins in living *Xenopus* oocytes. Comparison of ubiquitin derivatives and of two different forms of calmodulin suggested that signal intensities of in-cell HSQC spectra largely depend on protein–protein interactions of the target proteins inside cells. Furthermore, the maturation of a ubiquitin precursor derivative in living oocytes was observed. Our work demonstrates the potential usefulness of in-cell NMR with *Xenopus* oocytes for the investigation of conformations and functions of proteins under intracellular environmental conditions.

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