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Monitoring of the Refolding Process for Immobilized Firefly Luciferase with a Biosensor Based on Surface Plasmon Resonance

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In order to examine the possibility of the use of a surface plasmon resonance (SPR) sensor for real-time monitoring of the process of refolding of immobilized proteins, the refolding of firefly luciferase immobilized on a carboxymethyldextran matrix layer was analyzed. The SPR signal of the immobilized luciferase decreased after unfolding induced by GdnCl and increased gradually in the refolding buffer, while there was no signal change in the reference surface lacking the immobilized protein. The decrease in the SPR signal on unfolding was consistent with the difference between the refractive indices of the native and unfolded protein solutions. The effects of blocking of the excess NHS-groups of the matrix layer on the refolding yield were examined by means of an SPR sensor. The results were consistent with those obtained with the enzymatic activity assay, indicating that the changes in the SPR signal reflected the real-time conformational changes of the immobilized protein. Hence, an SPR biosensor might be used for monitoring of the process of refolding of immobilized proteins and as a novel tool for optimization of the refolding conditions. This is the first demonstration that SPR signal changes reflect the conformational changes of an immobilized protein upon unfolding and refolding.

Key words: firefly luciferase, immobilization, refolding, SPR sensor.

Recent optical methodologies for analyzing protein structures, *e.g.* circular dichroism (CD) (*1*), small angle X-ray scattering (*2*), *etc.*, have been used to analyze the process of refolding of unfolded proteins in solution. However, there have been few useful techniques for analyzing the conformational change of a protein captured on a solid surface. It has been reported that native protein molecules adsorbed on ultra-fine beads could be analyzed using the CD technique (*3*). But it was impossible to monitor the process of refolding of unfolded proteins immobilized on beads, since sedimentation of the beads occurred due to aggregation of the beads *via* the hydrophobic interactions between unfolded proteins with the high concentration required for the CD measurement (our unpublished data). Therefore, monitoring methods for the refolding process for unfolded proteins captured on a heterogeneous system, such as a gel matrix, have been limited to biological activity measurement.

Recently, a biosensor based on surface plasmon resonance (SPR) has been widely used for the analysis of biomolecular interactions (*4*). The SPR signal, expressed as an arbitrary resonance unit (RU), reflects a change in the refractive index at the surface of a sensor chip. The apparent refractive index is determined by the mass and dielectric properties of the substances. The SPR sensor has been mainly used to study protein/protein or protein/DNA interactions, in which a mass change according to association or dissociation among molecules was detected. Since the folding states of proteins will affect their dielectric properties, the SPR signal would include conformational information on immobilized proteins. This idea was first presented by Sota *et al.* (*5*), but they mainly analyzed the signal change between the baseline signal and the signal in the presence of an acidic buffer. However, it has become clear that the signal change observed under this condition mainly corresponds to the protonation of amino acid residues, rather than the conformational change (*6*).

In this study, we investigated the SPR signal changes of firefly luciferase immobilized on a sensor chip in a BIAcore apparatus in the presence of a refolding buffer after guanidine hydrochloride (GdnCl) treatment. We used firefly luciferase because we have already reported about the refolding of luciferase immobilized on agarose beads examined by means of an enzymatic the luminescence assay (*7*), and the results of this study could be compared with those previously reported. The surface of the SPR sensor chip with carboxyl groups and *N*-hydroxysuccinimide (NHS) groups for immobilization is similar to that of the agarose beads used in our previous study, which would justify the comparison of the results.

The BIAcore system, sensor chip CM5, Tween 20 and amine coupling kit containing NHS, *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide (EDC), and 1 M ethanolamine-hydrochloride, pH 8.5, were obtained from BIAcore AB (Sweden). Purified and lyophilized *P. pyralis* luciferase was

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Abbreviations: GdnCl, guanidine hydrochloride; NHS, *N*-hydroxysuccinimide.

purchased from Sigma, dissolved in deionized water, and stored at −20°C at a protein concentration of 1.0 mg/ml. Dithiotheitol (DTT), GdnHCl and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from WAKO (Osaka). All other chemicals were of analysis grade, and solutions were made with sterilized water.

Immobilization of firefly luciferase was carried out by injecting the reagents into the flow cell of a sensor chip according to the amine-coupling method. A continuous flow of HBS (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) at 5 μl/min was maintained during the immobilization period. Experiments were carried out at 25°C. In detail, the carboxymethyldextran matrix was activated by injection of 60 μl of a solution containing 0.2 M EDC and 0.2 M NHS. Next, 10–50 μl of firefly luciferase (100 μg/ml in 100 mM phosphate buffer, pH 6.0) was injected, followed by injection of 60 μl of 1.0 M ethanolamine to block the excess NHS-ester groups and injection of 10 μl of 2 M NaCl to wash out the non-specific binding molecules on the matrix. A immobilization level of 10,000–30,000 RU, corresponding to 10–30 ng/mm^2 of luciferase, was obtained by this procedure. On the other hand, the sensor surface of a reference lane was chemically modified by the injection of 1.0 M ethanolamine instead of the protein.

After the immobilization, the refolding buffer (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT) was applied as the running buffer at a continuous flow rate of 20 μl/min. Then the unfolding buffer (6 M GdnCl, 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT) was injected over 5 h (10 pulse injections of 30 min). The SPR signal decreased after each injection of the unfolding buffer (Fig. 1a). The level of decrease against that for the reference surface is defined the SPR signal change caused by unfolding (dRU). Within 5 h, dRU reached an equilibrium value, which meant the completion of unfolding of the immobilized luciferase. The injection was terminated by replacement of the unfolding buffer with the refolding buffer. There was no signal change in the reference lane between before and after injection of GdnCl, which indicated that the change in the SPR signal in the luciferase-immobilized lane reflected the change in the state of the immobilized protein (Fig. 1b). After termination of the unfolding treatment, the SPR signal of the protein-immobilized surface increased gradually with the continuous flow of the refolding buffer, while there was no signal change at the reference surface. Hence, this signal increase could be considered as the SPR signal change due to refolding of the immobilized protein.

Figure 2 shows that the SPR signal change on unfolding (dRU) is proportional to the amount of luciferase immobilized on the sensor surface with a fitting R value of 0.93. This result was compared to the refractive index change on unfolding measured with an ABBE refract meter (ABBE, Tokyo). Various concentrations of firefly luciferase were dissolved in the refolding buffer or the unfolding buffer. Luciferase was unfolded in the unfolding buffer, as described previously (*8*). The balance of the refractive index between a protein solution and a solvent reflects the presence of a protein. The difference between the balance of the native luciferase and the unfolded luciferase at the same protein concentration means a refractive index change of firefly luciferase caused by unfolding. Unfolding of firefly luciferase decreased the refractive index of the protein, which was likely due to the dielectric constant change; the absolute value of the refractive index change increased in proportion to the concentration of firefly luciferase (Fig. 2). We investigated the relationship between the decrease in the refractive index of the soluble protein upon GdnCl treatment and the decrease in the SPR signal of the immobilized protein. The scales for the x-axes (amount of immobilized luciferase and concentration of luciferase solution) and y-axes (SPR signal change on refolding and refractive index change on unfolding) in Fig. 2 were correlated according to the BIAcore AB manual; 30,000 [RU] protein immobilized on the sensor surface corresponds to 150 [mg/ml] of protein solution, and an SPR signal change of 10,000 [RU] corresponds a refractive index change of 0.001, respectively. The relationship between the decrease in the refractive index and the unfolded protein concentration is consistent with the linear relation given by the SPR signal change

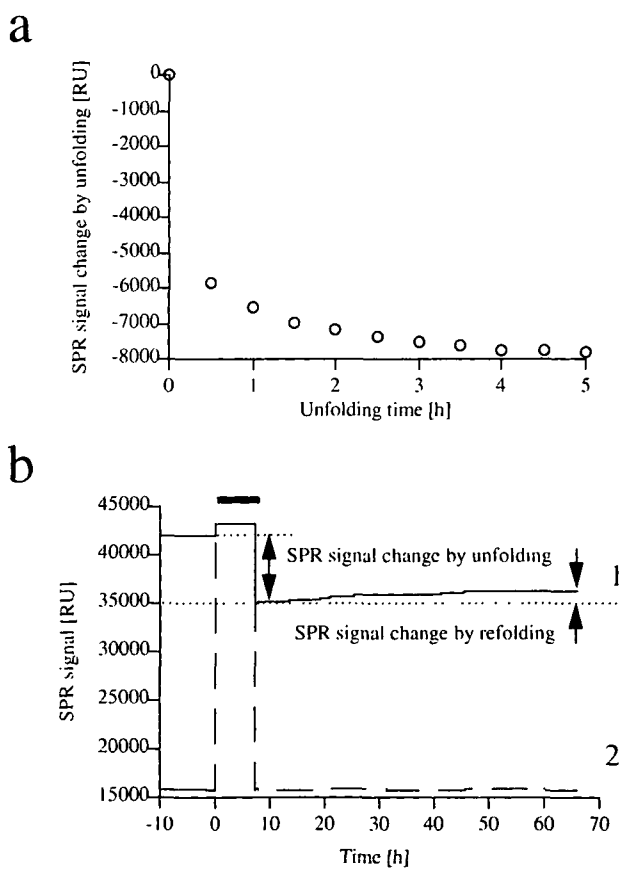

Fig. 1. **(a) SPR signal change of the luciferase-immobilized surface with unfolding treatment.** SPR signal changes from the preinjection level during the pulse injection of GdnCl are shown. Immobilized luciferase (30,000 RU) was treated with the unfolding buffer (5 M GdnCl, 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT) for 5 h. **(b) Typical SPR signal time courses of luciferase-immobilized (1: Solid line) and reference (2: Broken line) surfaces in responce to GdnCl injection.** The thick bar indicates the duration of injection of GdnCl. The SPR signal changes on unfolding and refolding are shown. After unfolding, the immobilized luciferase (30,000 RU) was refolded in buffer containing 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT. The flow rate was maintained at 20 μl/min. The temperature of the sensor surface was kept at 25°C.

(shown as a dashed line in Fig. 2) within the error of measurement for the refractive index (5×10^{-4}). So it might be concluded that the change in the SPR signal reflects the change in the refractive index of firefly luciferase caused by its conformational change.

After injection of the unfolding buffer and the resulting protein unfolding, the refolding buffer was supplied to the sensor chip and the refolding of the unfolded luciferase was initiated. It could be considered that the change from the unfolding buffer to the refolding buffer on the surface of the sensor chip occurs rapidly, since the response signal of the reference surface returns immediately to the pre-injection level, as shown in Fig. 1, b-2 (dashed line). After supplying the refolding buffer, the SPR signal increased gradually, which meant an increase in the refractive index of the sensor surface upon refolding of the immobilized luciferase (Fig. 1, b-1). The refolding yield was calculated on the basis of the recovered SPR signal as a percentage of the dRU. Since there was little difference in the refolding yields between three experiments involving changing of the amount of immobilized luciferase, the results of one of the three are shown in Fig. 3. Since there was no signal change in the reference lane in the refolding buffer, the increase in the SPR signal reflects the increase in the refractive index of the unfolded, immobilized luciferase in the refolding buffer, which might mean refolding of luciferase.

To confirm that the immobilized luciferase actually refolded after the increase in the SPR signal, luminscence activity on the sensor chip was measured directly. The same amount of luciferase was immobilized on two different sensor surfaces in the same sensor chip. 15,000 RU of luciferase was immobilized on both lanes 2 and 3, as described above. Unfolding buffer was only injected into lane 1 (reference lane) and lane 2. After injection of the unfolding buffer, the refolding buffer was supplied to lanes 1, 2, and 3. This enabled comparison of the enzymatic activity of the refolded luciferase (lane 2) with that of the native luciferase (lane 3). The luminescence activity of the immobilized luciferase was measured directly under a the microscope. Bright Glo Luciferase assay reagent (Promega, USA) was used as a substrate. 50 μl of Bright Glo was spotted on to the gold sensor surface directly and then the luminescence of each lane was observed at the same time. Photons were counted with an ARGUS-50 degital video microscopy system (Hamamatsu Photonics, Shizuoka). The area intensity of each lane was calculated using the area analysis command of the ARGUS-50 control software. After subtracting the intensity of the reference lane (lane 1), the

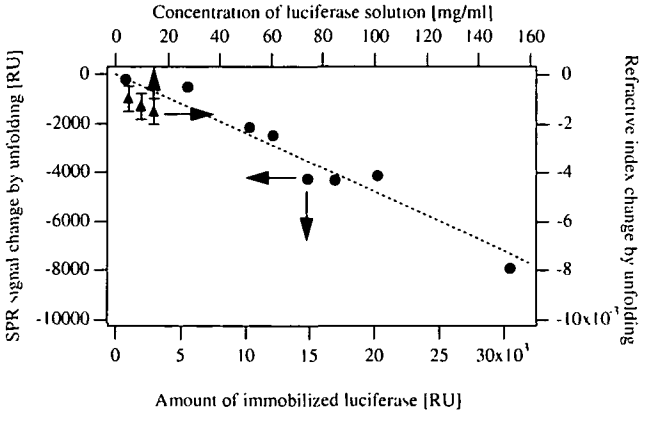

Fig. 2. **Correlation of the SPR signal change on unfolding with the refractive index change on unfolding.** For the refractive index change, the refractive indices of 5–15 mg/ml solutions of native luciferase in the refolding buffer (100 mM potassium phosphate, pH7.8, 1 mM EDTA, 1 mM DTT) and unfolded luciferase in the unfolding buffer (5 M GdnCl, 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT) were measured. The difference between the balance of the native luciferase and the unfolded luciferase from the solvents at the same protein concentration indicates the refractive index change of firefly luciferase caused by unfolding. The error of measurement for the refractive index (5×10^{-4}) is shown as an error bar. For the SPR signal change, immobilized luciferase (700–30,000 RU) was unfolded for 5 h in the unfolding buffer and the SPR signal change from the pre-injection level was used. All experiments were carried out at 25°C. Each *x*- and *y*-axis scale is adjusted. Symbols: circles, correlation of the SPR signal change on unfolding and the amount of immobilized protein; triangles, correlation of the refractive index change on unfolding and the concentration of the luciferase solution. Arrows indicate the correspondence of each symbol to the *x*- and *y*-axes.

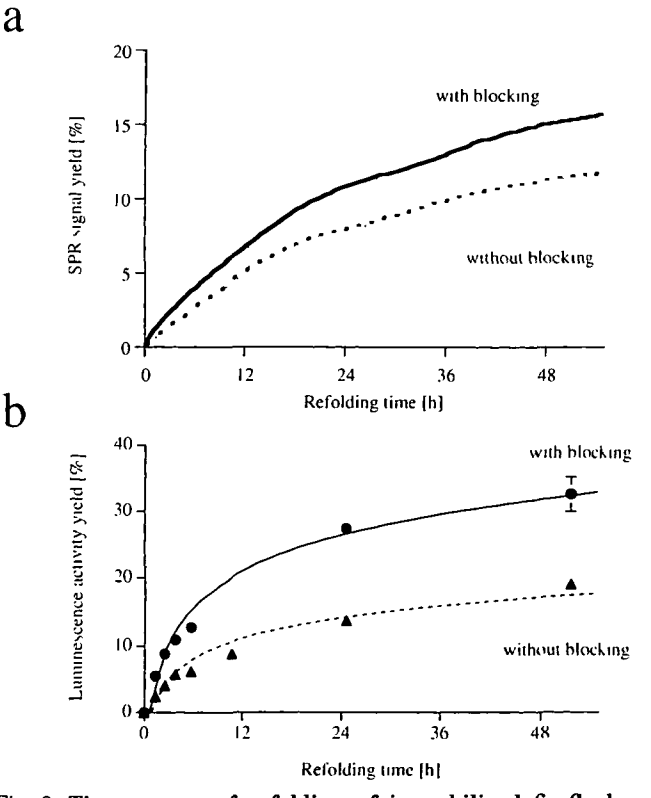

Fig. 3. **Time course of refolding of immobilized firefly luciferase with/without blocking of excess NHS-groups.** (a) Luciferase immobilized on the sensor surface (30,000 RU) was unfolded for 5 h in 5 M GdnCl, 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT. The refolding buffer contained 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT. The flow rate was maintained at 20 μl/min. The temperature of the sensor surface was kept at 25°C. (b) Luciferase immobilized on agarose beads (0.2 mg/ml-gel) was unfolded and refolded in the same buffer as described above. The luciferase acivity was assayed using a Luminescensor JNR (ATTO, Tokyo) after the addition of a 33% (v/v) gel suspension to the substrate solution, as described in our previous study (*6*). Symbols: circles (line), excess NHS groups were blocked with 200 mM ethanolamine; triangles (broken line), excess NHS groups were not blocked.

intensity of the refolded luciferase (lane 2) was compared to that of the native luciferase (lane 3) to calculate the refolding yield. After 72 h refolding, the sensor chip was removed from the BIAcore and the yield of recovered activity was calculated to be 17 ± 3% (mean value for two independent experiements). No luminescent activity was observed just after the unfolding treatment.

Next, the time course of the SPR signal yield of the luciferase immobilized on a sensor chip was compared with that of the luminescence activity yield of the luciferase immobilized on agarose beads using the same immobilization protocol (Fig. 3, a and b). The time course profiles are almost identical in Fig. 3, a and b, which validates the correspondence between the SPR signal and the luminescence activity. Refolding of soluble luciferase without molecular chaperones also takes several days, which is thought to be due to kinetic trapping of unfolding intermediates (*9*). This might also be considered as a reason for such slow refolding of the immobilized luciferase. In addition to this, there is the possibility that interactions between protein molecules and a matrix slow the refolding down, since we showed that such interactions could have a negative effect on the refolding of immobilized luciferase (*7*). The final yield of the SPR signal after 48 h (15%) was lower than that of the enzymatic activity (30%). This might be because refolding at the sensor surface was influenced by interactions with the matrix or because the luciferase concentration of the sensor surface (corresponding to 50–150 mg/ml) was much higher than that of gel beads (0.2 mg/ml). This high concentration is, however, inevitable with the BIAcore assay in order to obtain effective signal changes.

For further validation of the BIAcore, the effect of blocking of excess NH_2-sensitive NHS groups on the refolding yield was examined, as was previously done for luminescence activity measurement (*7*). Without blocking of excess NH_2-sensitive NHS groups, the NHS groups are hydrolyzed and carboxyl groups are exposed. The refolding yield of the SPR signal of the sensor surface with blocking was higher than that without blocking (Fig. 3a). This result is consistent with the result (Fig. 3b) obtained on luminescence activity measurement (*7*).

These results indicate that an SPR sensor can monitor the conformational change on the refolding of an immobilized protein *via* its SPR signal change. This idea was justified by the consistency of the SPR signal change upon unfolding with the refractive index change, and that of the SPR signal change upon refolding with the luciferase activity change. Although the yield of the SPR signal was not quantitatively equal to that of the enzymatic activity, observation of the effectiveness of blocking of the excess NHS groups in the immobilization procedure on the yields of both the SPR signal and enzyme activity also supports this idea.

The SPR sensor has a potential as a novel tool for monitoring the conformational changes of immobilized proteins since there has been little methodology for detecting the conformational behavior of immobilized proteins so far. The SPR sensor is very effective since it is so highly sensitive that only a little protein (less than μg) is required. In addition to this, it would be useful for proteins whose biological activities are difficult to measure. So it would be useful for optimization of efficient refolding conditions. Further studies to validate this idea are in progress.

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