Rapid Communication *J. Biochem.* **127**, 351–354 (2000)

Refolding of Firefly Luciferase Immobilized on Agarose Beads

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Received September 27, 1999; accepted January 11, 2000

The renaturation yield of the denatured firefly luciferase decreased strongly with increasing protein concentration in a renaturation buffer, because of aggregation. In this study, firefly luciferase was immobilized on agarose beads at a high concentration. Although the protein concentration was extremely high (about 100-fold) compared to that of soluble luciferase, the renaturation yield was comparable with that for the soluble one. Thus, immobilization was shown to be effective for avoiding aggregation of firefly luciferase. It was also shown that the optimum buffer conditions for renaturation of the immobilized luciferase were the same as those for the renaturation in solution. Also, it was indicated that electrostatic interactions between a protein and the matrix have a negative effect on renaturation of the immobilized luciferase since the renaturation yield decreased at acidic pH only for the immobilized luciferase. These novel observations are described in detail in this paper.

Key words: agarose beads, firefly luciferase, immobilization, renaturation.

Firefly luciferase, from the North American beetle *Photinus pyralis*, is a 61-kDa monomeric protein responsible for the production of light through conversion of chemical energy into visible light. Luciferase catalyzes the Mg^{2+}-ATP–dependent oxygenation of the heterocyclic component firefly luciferin, ultimately yielding an electronically excited oxyluciferin species. This excited state product then returns to the ground state with the emission of yellow-green light.

Because the enzymatic luminescence assay is highly sensitive, with a detection limit of about 10^{-18} mol of the enzyme, luciferase has become a popular model for studies on protein refolding *in vivo* or *in vitro* (*1–6*). When chemically denatured or heat-inactivated luciferase was diluted with reticulocyte lysate or wheat germ extract, the enzyme could be reactivated in minutes and with yields of 60–80% when ATP was provided by a regenerating system (*2–6*). In contrast, the renaturation of luciferase in a buffer lacking cellular components was insufficient, and the enzyme was found to aggregate during refolding (*1, 2, 7*). Recent investigation revealed that only at a low protein concentration (2–5 μg/ml) and low temperature (10°C), conditions chosen to minimize aggregation as a competing side reaction, could firefly (*P. pyralis*) luciferase recover about 65–80% of its native enzymatic activity without cellular factors (*7*). The renaturation yield, however, decreased dramatically with increasing protein concentration. For scale-up to an industrial level, renaturation with a low protein concentration is not preferable from the viewpoint of cost for large facilities, large amounts of detergents and solutions having to be used.

The immobilization of proteins on a solid support is an effective method for the prevention of aggregation during renaturation *in vitro*. This approach has been shown to be useful in the renaturation of a number of proteins (*8–14*). The advantage of immobilization is that aggregation is avoided, which allows renaturation at a high protein concentration, resulting in a higher yield per volume ratio. The purpose of this paper is to show that the phosphate buffer, which was shown to be effective for the renaturation in solution, is also effective for the renaturation of immobilized firefly (*P. pyralis*) luciferase on agarose beads, and that immobilization is an effective approach for the renaturation of luciferase at a high concentration from the viewpoint of prevention of aggregation.

Purified, lyophilized *P. pyralis* luciferase was purchased from Sigma, dissolved in deionized water, and stored at –20°C at a protein concentration of 1.0 mg/ml. The gel employed was NHS-activated Sepharose 4EF from Amersham, Pharmacia Biotech Inc. (Sweden). Substrate luciferin was from Sigma. Dithiothreitol (DTT) and guanidine hydrochloride (GdnCl) were purchased from WAKO (Osaka). All other chemicals were of analysis grade, and solutions were made up in distilled water.

The immobilized luciferase was prepared by the standard procedure recommended by Pharmacia with a slight modification. The NHS-activated Sepharose 4EF gel was washed with 10 ml/ml–settled gel of 1 mM HCl to remove stabilizers and then washed again with 10 ml/ml–settled gel of the coupling buffer (0.2 M $NaHCO_3$, pH 8.3, 0.5 M NaCl). Several volumes ranging from 0.01 to 0.5 ml of the firefly luciferase solution were added to 5 ml of the gel suspension containing 20% (v/v) gel in the coupling buffer to obtain immobilized firefly luciferase. The mixture was stirred gently for 12 h at 4°C and then filtered. The immobilized firefly luciferase gel was then thoroughly washed with the coupling buffer. The remaining NH_2-sensitive

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

NHS groups on the gel were desensitized by hydrolysis in 200 mM ethanol amine buffer, pH 8.5, for 4 h at 4°C. Again, the gel was washed with a storage buffer (100 mM sodium phosphate buffer, pH 7.8, 1 mM EDTA) and stored at 4°C. The amount of immobilized protein was estimated from the balance of introduced and leached firefly luciferase. No sign of protein leaching was detected on SDS gel electrophoresis and silver staining in the enriched solutions of the filtrates and the coupling buffers used for washing. This indicates that the introduced luciferase was totally immobilized on the Sepharose gel at a concentration ranging from 0.01 to 0.5 mg/ml-gel.

The immobilized luciferase was denatured by stirring gently in a 6 M GdnCl solution for 12 h at 4°C. The denatured luciferase possessed no enzymatic activity. For effective renaturation, the denatured immobilized luciferase was filtered and washed with about 10 ml/ml–settled gel of a renaturation buffer (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT). This is the same buffer as that optimized for the renaturation of soluble firefly luciferase (7). The 33% (v/v) gel suspension in the renaturation buffer was stirred gently at 4°C. To measure the rate of refolding, a certain amount of the gel suspension was taken at appropriate time intervals and then subjected to the enzymatic activity assay.

The immobilized luciferase activity was assayed using Luminescencer JNR (ATTO, Tokyo) at 25°C. A fluorescence microcuvette was filled with luciferin and ATP reagents. The addition of the 33% (v/v) gel suspension started the light emitting reaction. The reaction mixture contained 100 mM tricine buffer, pH 8.0, 3 mM $MgSO_4$, 280 μM luciferin, 55 μM coenzyme A, and 55 μM ATP. The light emission intensity was recorded with a luminometer for 20 s. Correction for auto inactivation of luciferase was performed as reported by Herbst *et al.* (7). The renaturation yield was evaluated on the basis of the enzymatic activity as a percentage of that of control samples, that were handled identically but without denaturation. Each experiment was performed three times and the resulting average values were plotted.

The correlation of the concentrations of immobilized luciferase and the renaturation yields after 72 h is shown in Table I. There were no differences in the renaturation yields among the concentrations of immobilized luciferase examined here, indicating that interactions among denatured molecules at a high protein concentration may not occur, because the yield should dramatically decrease if interactions occur. At the concentration of 0.5 mg/ml-gel, however, intermolecular interactions among denatured molecules might be possible if the protein molecules are linearized in the denatured state. The reason why intermolecular interactions among denatured molecules did not occur even at the high concentration would be that the matrix of the beads interfered with the intermolecular interactions and/or that interactions between the matrix and protein molecules had a strong influence on the renaturation yield. The immobilized luciferase, 0.2 mg/ml-gel, was used in the following experiments.

In order to determine whether or not the conditions for renaturation are also optimum for the renaturation of immobilized luciferase, the renaturation yields under various conditions were estimated; (i) various concentrations of potassium phosphate (0, 20, 100, 200, 500, and 1000 mM), pH 7.8, 1 mM EDTA, 1 mM DTT, 2) 100 mM potassium phosphate of various pHs (6, 7, 7.8, and 9.2), 1 mM EDTA, 1 mM DTT, and (iii) various concentrations of NaCl (0, 20,

TABLE I. **Bound amounts and renaturation yields of immobilized firefly luciferase on agarose beads.**

Bound luciferase (mg/ml-gel)	Renaturation yield (%)
0.010	48
0.050	54
0.10	47
0.20	45
0.50	48

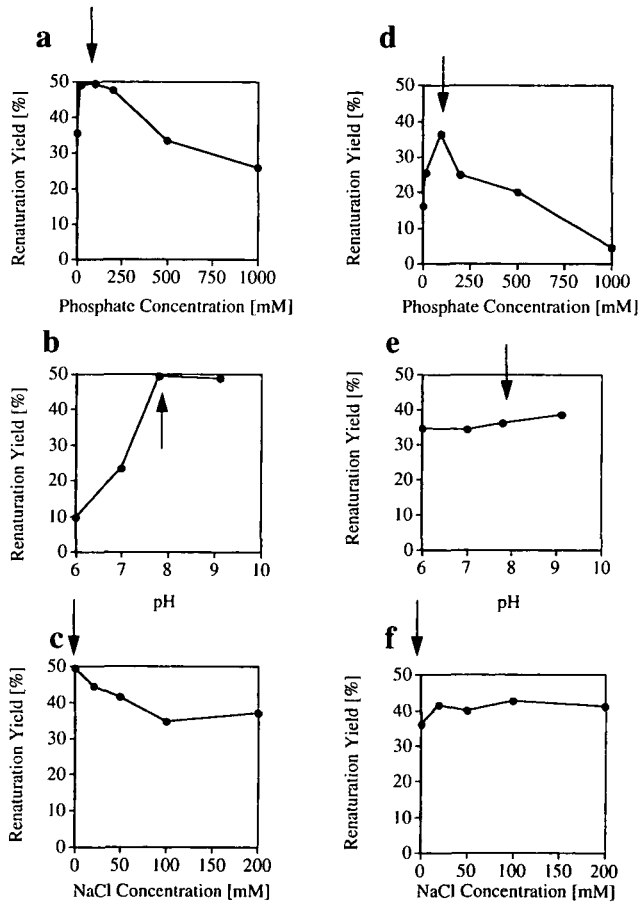

Fig. 1. **Renaturation yields of immobilized firefly (*Photinus pyralis*) luciferase (a, b, c) and the luciferase in solution (d, e, f) in various phosphate buffers.** The corresponding buffers are identical (a, d: various concentrations of potassium phosphate (0, 20, 100, 200, 500, and 1000 mM), pH 7.8, 1 mM EDTA, 1 mM DTT; b, e: 100 mM potassium phosphate of various pHs (pH 6, 7, 7.8, and 9.2), 1 mM EDTA, 1 mM DTT; c, f: various concentrations of NaCl (0, 20, 50, 100, and 200 mM) added to 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT). Arrows in the figure indicate the standard buffer conditions used by Herbst *et al.* for the renaturation of luciferase in solution (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT). Immobilized luciferase (0.2 mg/ml-gel) was denatured with 6 M GdnCl at 4°C for 12 h and then renatured in the renaturation buffers at 4°C for 72 hrs. Soluble luciferase (0.3 mg/ml) was denatured with 5 M GdnCl at 4°C for 1 h and then renatured at 4°C for 20 h after 100-fold dilution with the renaturation buffers. The renaturation yields were determined as percentages of the enzymatic activity of renatured samples against control samples, which were handled identically but without denaturation.

50, 100, and 200 mM) added to 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT. Phosphate buffer is better since the immobilized luciferase was stable in every phosphate buffer described above, whereas loss of enzymatic activity was observed in other buffers, for example, Tris or Hepes buffer (data not shown).

Figure 1 (a, b, c) shows the renaturation yields of the denatured immobilized luciferase after 3 days incubation at 4°C under the various conditions. The arrows in the figure indicate the optimum conditions for renaturation of the immobilized luciferase, *i.e.* 100 mM phosphate, pH 7.8, 0 mM NaCl.

Although Herbst *et al.* used 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT for the renaturation of soluble luciferase, no other conditions were examined as they were in this study, since they emphasized the effectiveness of the low protein concentration and low temperature for the renaturation process (*7*). We estimated the renaturation yields of soluble luciferase under the same buffer conditions. 300 μg/ml of luciferase was denatured in a buffer containing 5 M GdnCl, 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, and 1 mM DTT, as described previously (*7*). Renaturation was initiated by 100-fold dilution with various phosphate buffers as for the immobilized luciferase at 4°C. For the enzymatic assay, 10 μl aliquots were taken and mixed into the reaction mixture.

Figure 1 (d, e, f) includes the renaturation yields after 20 h incubation at 4°C under the different buffer conditions, which were also examined with the immobilized luciferase. Further incubation reduced the activity of the luciferase (data not shown). As for the concentrations of potassium phosphate and NaCl, the optimum concentrations are almost the same as those for the immobilized luciferase (a *vs.* d, c *vs.* f). On the other hand, the pH preferences are different. At lower pH (*6*, *7*), the renaturation yield of immobilized luciferase is low, whereas the yields of soluble luciferase are almost the same at various pHs. This indicates that the decrease in the renaturation yield at low pH is unique to the immobilized luciferase. This might be because of the interaction between carboxymethyl dextran and luciferase. Since the pK_a value of luciferase is about 6.4, luciferase becomes positively charged at pH 6, compared to at pH 7.8 and 9.2. On the other hand, carboxyl groups are negatively charged at all the pHs examined here since the pK_a of carboxyl groups is about 4. Then it could be deduced that the negative–positive electrostatic interaction between the Sepharose matrix and luciferase has a more negative effect on the renaturation yield than a negative–negative interaction. At pH 7, at which luciferase is positively charged but still has a more negative charge than at pH 7.8 and 9.2, the negative–positive interaction would decrease the renaturation yield. A decrease in the renaturation yield at acidic pH owing to electrostatic interactions between the matrix and protein molecules was firstly demonstrated in this study.

Figure 2 shows the time course of the renaturation yield of the denatured luciferase immobilized on Sepharose gel at 4°C in the renaturation buffer. Within the first 24 h, the renatured sample exhibited 26 ± 0.7% of the luminescence activity of controls. Upon further incubation for 2 days, the renaturation yield increased to 51 ± 5%. The renaturation yield was slightly lower than the reported value (65–80%) for the renaturation of soluble luciferase in the same buffer (*7*). This may be because interactions between the immobilized luciferase and the matrix (even a negative–negative one) have a negative effect on protein refolding. But it should be noted that the protein concentration corresponding a 50% renaturation yield (>0.5 mg/ml-gel) is at least 100-fold higher than that for the renaturation of soluble luciferase (5 μg/ml) (*7*). Also, the protein concentration is about 100-fold higher than that for the renaturation with cellular factors (2–5 μg/ml) (*1*, *2*, *6*).

The effect of desensitization of the excess NH_2-sensitive NHS groups on the renaturation yield was determined by comparing the renaturation yield for the gel whose actives sites were desensitized with that for the gel without desensitization. Without desensitization of the excess NHS groups, NHS groups are hydrolyzed and carboxyl groups are exposed. Figure 3 indicates that the time course of the renaturation yield for the gel with desensitization was about 10% higher than that for the gel without desensitization. This indicates that the interactions of the exposed carboxyl groups have a negative effect on the renaturation of denatured luciferase immobilized on the gel. To our knowledge, this is the first demonstration of the effect of desensitization of excess NHS groups on the renaturation of an immobilized protein. This is consistent with the finding of the negative effect of negative–positive interactions described above.

This paper indicates that the phosphate buffer is effec-

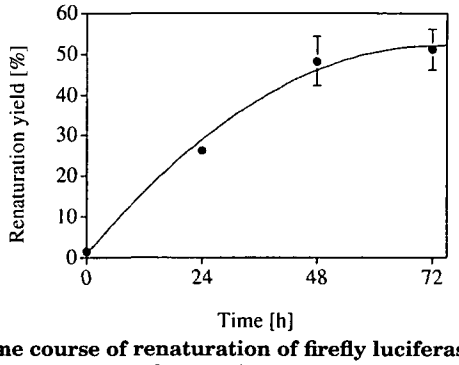

Fig. 2. **Time course of renaturation of firefly luciferase immobilized on agarose beads.** Immobilized luciferase (0.2 mg/ml-gel) was denatured with 6 M GdnCl at 4°C for 12 h. The renaturation buffer contained 100 mM potassium phosphate, pH 7.8, 1 mM EDTA, and 1 mM DTT, and renaturation was carried at 4°C.

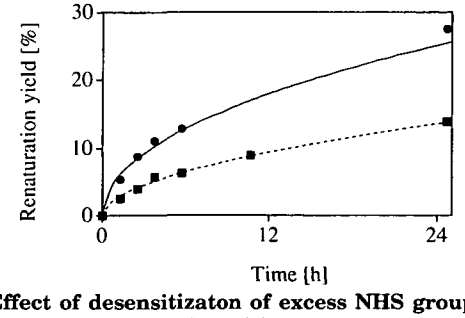

Fig. 3. **Effect of desensitizaton of excess NHS groups on the renaturation yield.** Immobilized luciferase (0.2 mg/ml-gel) with/without desensitization of the excess NHS groups was denatured with 6 M GdnCl and then renatured in the renaturation buffer at 4°C. Symbols: Circles, excess NHS groups were desensitized with 200 mM ethanol amine; squares, excess NHS groups were not desensitized.

tive for the renaturation of immobilized luciferase, as shown for the renaturation in solution (7). Immobilization of luciferase allowed renaturation at protein concentrations of more than 500 μg/ml with a yield of 50%. The protein concentration for refolding in solution corresponding to a yield of 50% was around 5 μg/ml (7). Thus, the protein concentration during renaturation could be increased 100-fold upon fixation of a protein to a solid support. This may be because interactions among denatured molecules are suppressed by immobilization. Hence, immobilization is an effective approach for the renaturation of a protein which aggregates easily at a high concentration. In addition to this finding, it was also shown that electrostatic interactions between the matrix and luciferase had an negative effect on renaturation, especially negative–positive ones. Although it is not clear whether or not this is also applicable to other immobilized proteins, it could be worth considering for improvement of the renaturation yield of an immobilized protein.

We thank Mr. Teruhisa Mannen and Mr. Satoshi Yamaguchi for many valuable discussions.

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