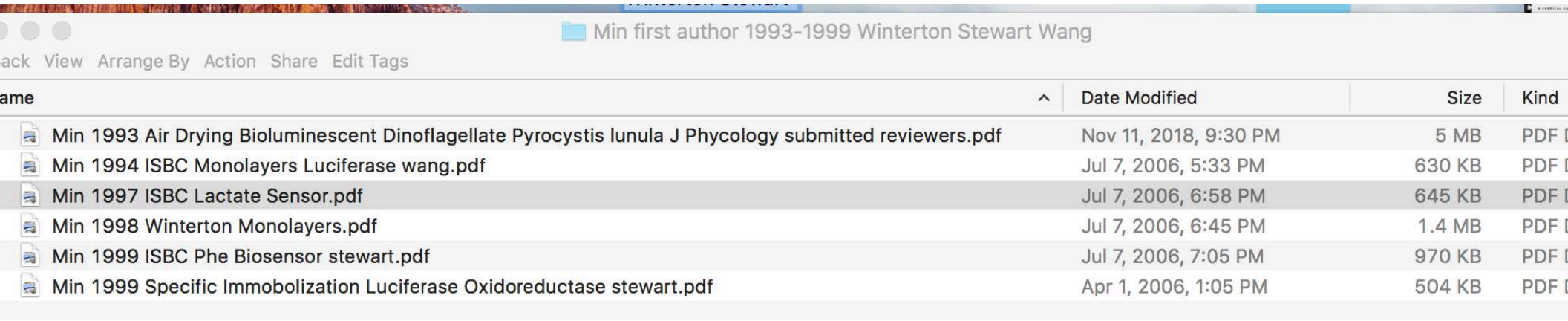
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February 28, 1994

Dr. Joseph D. Andrade
Department of Bioengineering
2480 MEB
University of Utah
Salt Lake City, UT 84112

Dear Dr. Andrade,

Enclosed are two reviews of your manuscript (#93-196) on "Air drying a bioluminescent dinoflagellate (*Pyrocystis lunula*): feasibility study."

The reviewers seem to agree that the paper addresses a relevant question. However, there are a number of problems that need to be solved before the paper can be published. Both reviewers note the potential for toxicity upon drying down as the salinity and/or DMSO is concentrated during evaporation. Reviewer #1 indicated that it would have been more reasonable to start with lower concentrations of additives. Additional experiments would be helpful; at the very least, this potential problem needs to be mentioned. Reviewer #2 cited a number of technical problems and called for more in-depth explanation of methods. I also read the manuscript (my comments are in blue; red on the cover sheet) and found a number of concerns that must be satisfactorily addressed:

1. Please use *Journal of Phycology* format throughout; use a recent issue as a guide.

2. There is a range of extremes in how some aspects are handled. Part of this may come from an unfamiliarity with how algal research is communicated. At one extreme is the description of dinoflagellates on page 3. The vast majority of readers know what dinos are. This sort of description would be appropriate if you were writing something general for student use, but not for professionals. On the other hand, your description of some of the possible reasons for your results (e.g. on page 6) is much too detailed for the evidence at hand. Your results are fairly simple observations; they were not conducted at the molecular/cellular levels. Therefore, speculation about phase transitions, lipid reorganization, and the like are out of place and inappropriate without better reasons for making the linkage. Some general statements might be in order, but without adequate justification and relevance to the actual results, such detail must be deleted.

3. You may have felt obliged to add the detail on mechanisms to fill out the paper, because very little of the text is spent in describing your actual results. Virtually nothing is said about Figure 5, which contains most of the data for the paper. Your description of cell morphologies is

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very elementary and pretty much confined to the figure legends. This is not appropriate; you need to describe the cells in a sequential order in the text so that the reader can easily make the necessary comparisons.

4. Again, there are extremes in how methods are presented. You provide two digrammatic figures showing the normal versus slow air drying processes. Neither of these figures is needed because your written descriptions in the Materials and Methods are adequate in portraying the steps of the procedures. On the other hand, you provide no information on other critical pieces of information, such as length of slow drying, number of times the experiments were repeated, how viability was actually measured (i.e. how many cells recovered to get a rating of viable), etc.

5. The descriptions of the cell morphologies are inadequate. What are the spine-like projections at the ends of the cells in Figure 4b? In that figure more than just the cell membrane appears to be broken. In fact, it is impossible to see a "broken" cell membrane. The cell contents appear to be disorganized and dispersed in contrast to Figure 1. Furthermore, what do you mean by cell membrane? Cell membrane can be a general term for all membranes in the cell. Most botanists would probably use plasma membrane or plasmalemma for the outermost membrane of the cell. Is that indeed what was broken? How can the reader tell this by looking at the figures?

6. Figures 1, 4, and 6 should be reduced, trimmed and arranged and mounted on a single plate according to *Journal* format. A plate of halftones should be no more than 17.2 cm in width (two-column) or 8.5 cm (one column).

7. A number of additional questions and inconsistencies are marked on the manuscript.

In summary, this paper needs a major rehaul before it can be published. There is no question that the subject is an important one. The storage and availability of viable cells for teaching and research would be a significant contribution. But, the premise on which you base viability is only weakly supported (observation of bioluminescence) if you do not describe how you established the quantitative criteria for viability. Was bioluminescence in only one cell sufficient to deem the culture viable? In 50% of cells? In 70% of cells? In addition, the paper needs to be greatly trimmed; speculation regarding mechanisms needs to be eliminated or greatly reduced so that it clarifies rather than muddies the water. You do not need filler. The manuscript can be very short (published in NOTE format) and still be a significant contribution. Your findings as presently configured do not merit a long document. Figures can be deleted. The reviewers' comments need to be addressed.

If you choose to continue here, please return the **enclosed blue-marked copy**, a revised original plus one copy, the original figures, and your responses to the reviewers' comments, point by point, at your earliest convenience. I will read the revision and determine at that time whether it is acceptable for publication.

If you would like to submit a color or black and white photo for consideration as a *Journal* cover, please see the enclosed form.

You still have much work to do, but the result should be a much stronger contribution to the literature.

Sincerely,

Carole A. Lembi

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Rev. #1

Air Drying a Bioluminescent Dinoflagellate (Min and Andrade)

This is a reasonable paper with a reasonable objective, though without very positive results. The commentary on mechanistic considerations is particularly good, but certain basic points seem to have escaped the authors. They should consider the following questions, perhaps in their discussion of future directions for any follow-up work.

1. Direct exposure of their cells to high concentrations of additive may induce unnecessary osmotic injury. For example, it would be less damaging to expose cells to 1 M DMSO by first treating them with 0.25 M DMSO and then transferring them into 1 M DMSO.

2. However, since the objective of their research is to dry the cells, the logic of using an agent like DMSO at a concentration like 1 M seems faulty. If the volume of water in the solution is reduced by evaporation, such that the final liquid volume is brought to 10% of the initial liquid volume, then the concentrations of all dissolved solutes will rise by 10-fold, including solutes such as DMSO. Thus, 1 M DMSO (about 7% by volume) would become 10 M (about 70% by volume) after drying, a presumably fatal concentration. Even 1 M DMSO exposed to cells for 3 days under ordinary conditions, let alone under conditions of stress such as drying, would be expected to be very toxic. Thus, 0.01 M DMSO might have been a better choice.

3. What is meant by "never allowed to become completely dry"?

4. Bioluminescence and morphology may be reasonable things for students to observe, but may not represent viability. The ability of the cells to divide would unequivocally represent viability.

5. "Bound water" is a misnomer: there is no such thing!

6. I do not believe Figure 6 shows cells dividing: the figure looks to me more like cells that have fused together.

7. The authors ought to consider the possibility of genetically modifying their organisms to allow them to elaborate intracellular solutes that have been shown to protect against dessication in organisms that are naturally resistant, or permeabilizing the organisms to make them take up such solutes.

Rev. #2

Review of the manuscript "Air drying a bioluminiscent dinoflagelate (*Pyrocystis lunula*): feasibility study"
(by Min and Andrade, 1994)

This paper deals with a new and simple procedure to preseve viable microalgae for short periods of time, a very little developed field in applied phycology. At this regard, the paper may be considered as a relevant contribution. It also contains a complete set of references from which a clear discussion of biological processes occurring during desication is compiled. There exists however many doubts respecting to the method used to dry the algae. The main problem that needs clarification is the following:

Two drying methods are described:

a)-The NORMAL drying, where cultures and solutions are let to dry for three days.

b)-The SLOW drying, where cultures are filtered through filter papers and then the solutionn is also filtered.

In case a) the evaporation at room temperature is concentrating both algae and salts, something that does not happens in b). On rehydration with new medium in artificial sea water, a very high salinity may be achieved in a). These salinities must be given in the paper. In addition , some controls for viability and bioluminiscence should be also stressed for undryed algae under such salinity conditions.

The specific questions and comments are marked on red on the manuscript and their corresponding descriptions are listed below.

1) Author's name has to be given.

2) What happened after three days? How concentrate was the algae suspension? How was the salinity?

3) As stated above, after rehydration, what salinity was achieved and how can this affect to viability and bioluminiscence of Pyrocystis lunula?

4) What type of filter paper (cellulose, fiber glass.....)?

5) How long were the algae exposed to the additive solutions. It is not clearly described whether the additive solutions were also filtered or not. Anyway, it seems to be difficult to pass through the filter if they are already blocked by the cells.

6) If wet filter papers were tightly covered, how was the degree of drying? An estimation of it should be made.

7) What does this means? Please quantify the amount of water and solute concentration.

8) Absence of viability is very likely to have occurred because the extended exposure to increasing osmolalities (up to an unknown level but important to know) during the normal drying system. From the first paragraph of Results and Discussion a misleading conclusion is obtained since only initial additive concentrations are considered and their build up produced by evaporation is not taken into account.

9) stresses destabilizing.

10) How can DMSO improve trehalose penetration. Please explain briefly.

11) Not neccesary. Repetitive.

12) Which methods? Please refer.

13) This is a very important fact.

14) Why "seemed" to be toxic?

15) After how long exposure?

16) In Fig. 5, the recoveries shown for all additive combinations tested are not quantified. Thus, this Figure is of poor usefulness since the relevance of the method is going to relay on significant recoveries. Many authors state 50% as the minimum recovery to consider the method suitable. It is essential to know percentage recovery after rehydration.

How many replicates did exist per treatment in Fig. 5?

#2

Air Drying a Bioluminescent Dinoflagellate (Pyrocystis lunula): Feasibility Study[1]

Dong J. Min and Joseph D. Andrade[2]

Department of Materials Science and Engineering and
Department of Bioengineering, 2480 MEB
University of Utah, Salt Lake City, UT 84112 USA

Running title: Air Drying of Dinoflagellate

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1. To whom correspondence should be addressed.

[1] Received 22 December 1993.
[2] Address for reprint requests.

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ABSTRACT

A marine bioluminescent dinoflagellate has been subjected to normal and slow air drying processes. We have used the preservation additives trehalose, sucrose, polyethylene glycol (PEG), and dimethyl sulphoxide (DMSO) to attempt to minimize desiccation damage. The normal air drying process produced highly altered cells with no bioluminescence after rehydration. Slow air drying produced more positive results; dinoflagellates in 0.1M DMSO, 1% PEG, and 5% PEG survived upto 3 days after drying as assayed by rehydration and bioluminescence. We believe it is possible to further develop an air drying process for these organisms.

Key words: air drying, bioluminescent, desiccation, dimethyl sulphoxide, dinoflagellate, polyethylene glycol, trehalose

INTRODUCTION

The preservation of cells, plants, animals, and foods has been studied for a long time. The two most common storage methods are drying and freezing. The common drying methods are air drying, vacuum drying, and freeze drying. ~~There is now growing~~ interest in air drying because it is cheaper and simpler than the other dry processes.

Certain plants, like the desert Selaginella lepidophylla (resurrection plant), are able to dry out completely during summer droughts and then come back to life upon rehydration without apparent damage ~~(1)~~. Other examples of desiccation tolerance include pollen, ferns, mosses, yeasts, tardigrades, and algae (2). Such organisms generally contain large amounts of trehalose, ~~a~~ an α-disaccharide of glucose ~~(2, 3)~~. Trehalose synthesis and accumulation apparently ~~avoids~~ prevents desiccation damage (1, 3, 12, 16, 19, 21, 22, 23). Other protectants against desiccation include sucrose, polyethylene glycol (PEG), and dimethyl sulphoxide (DMSO). Those chemicals are also well known as cryoprotectants (4, 5, 6, 15).

Sucrose is commonly found in seed embryos and is a common cell osmolyte in many unicellular algae, certain salt-tolerant plants, and many insects (2, 7). PEG is a good cryoprotectant for some proteins and was more inhibitory to yeast growth than was sucrose at a similar water activity (5, 6, 9). As aqueous solutions of PEG are hospitable to living cells, it is often utilized in tissue culture media and for organ preservation (27).

Use journal format: (Roser 1991)

Leopold 1990, (Rowe et al. 1992)

What is the significance of this?

DMSO is used as a penetrating additive because it penetrates membranes and enters both plant and animal cells (4, 14). Morris found that using an additive with a high permeability is not as damaging on an osmotic basis as one with lower permeability (14).

We studied the combination of trehalose and DMSO, hoping that ~~the~~ normally impermeant penetrating additives such as trehalose? will be taken up more rapidly in the presence of DMSO (14).

We investigated the air drying of a marine dinoflagellate, a phytoplankton. Many marine dinoflagellates are bioluminescent, producing a blue light when stimulated at night (18). Dinoflagellates are single-cell organisms with both animal and plant-like characteristics, generally multiplying by cell division (10).

Well known by readers

The best additives for air drying depend on the properties of the organism. We wanted to study the air drying of single-cell dinoflagellates to hopefully minimize costs involved in the shipment of such organisms. We have developed dinoflagellate cultures which provide teachers and students with the experience of bioluminescence. To accomplish this we have to deliver the dinoflagellates within 3 days in order to keep the cells alive. Dry cells which could be easily reconstituted would greatly simplify the shipping problems. In this study we ~~chose~~ investigated the survival of the bioluminescent dinoflagellate Pyrocystis lunula after two types of air drying, normal and slow...?

Rewrite — something like this.

MATERIALS and METHODS

The dinoflagellate used in these experiments was *Pyrocystis lunula* (Fig.1). The basic Guillard's F/2 culture media consisted of 0.5 ml/l vitamin working stock, 1 ml/l major elements, and 1 ml/l trace metals in artificial sea water (8). The dinoflagellates were maintained at 20 °C with a 12/12h light/dark cycle.

The normal air drying process is shown in Figure 2. Dinoflagellate cultures were mixed with the additive solutions and then air dried in an open petri dish at room temperature for 3 days.

The dried cells were rehydrated in culture medium and evaluated for growth and bioluminescence activity. The solutions used were 0.1M, 0.5M, and 1M trehalose (Sigma Chem. Co.); 0.1M, 0.5M, and 1M sucrose (Mallinckrodt Specialty Chem. Co.); 1%, 5%, and 10% PEG (average Mol. wt.=3400, Sigma Chem. Co.); and 0.1M, 0.5M, and 1M DMSO (Mallinckrodt Specialty Chem. Co.). We used culture media as an aqueous buffer for the solutions.

The slow air drying process is shown in Figure 3. The cultures were first concentrated on filter paper. The filtration step was repeated several times to obtain high amounts of dinoflagellates. The additive solutions were poured slowly onto the dinoflagellates on the filter paper, thus bringing the dinoflagellates into contact with the solutions. After completely removing the excess solution, the wet filter paper with dinoflagellates was dried slowly in a petri dish covered tightly with plastic wrap for the desired time at room temperature. The same light cycle was maintained throughout

the "drying" process. The cells were never allowed to become completely dry. After storage, the partially dried dinoflagellates on the filter paper were rehydrated in culture media. The solutions used were the same as for normal air drying.

The resuspended cells were evaluated by their bioluminescence activity and by study of morphology under the optical microscope. Viable dinoflagellates could be recognized by their blue bioluminescence and by their morphology.

How was bioluminescence activity measured? Visually? If so, how many cells (or %) had to be bioluminescent in order to be rated as survivors?

RESULTS and DISCUSSION

The normal air drying process (Fig.2) produced ~~very~~ altered cells with no bioluminescence ~~after exposure to all in any of the solutions and~~ after rehydration. ~~The morphology of the cells during drying is shown in Figure 4.~~ After drying for 2 days in 0.1M trehalose, the cells had shrunk (Fig.4(a)). ~~Also in the case of drying for~~ 2 days in 0.1M trehalose mixed with 0.1M DMSO, ~~we can see broken~~ cell membranes appeared (Fig.4(b)). With the higher concentrations of trehalose and trehalose mixed with DMSO, and the solutions of sucrose, PEG and DMSO, ~~we obtained similar results.~~

Water is a key component in maintaining the structure of membranes, nucleic acids, and proteins (11, 12). The three-dimensional structures of many biological molecules depend on the stabilization of the hydrogen bonding between water and macromolecules (1, 11). Membranes also depend on this complex bonding; the preservation of membrane interactions and structure is very important in research on desiccation. The preservation of microorganisms is closely related to maintenance of cell membrane structures during dehydration and rehydration. Desiccation of many microorganisms leads to large volume changes and metabolic unbalance due to changes in solute concentration, which occur during drying (2, 4). The cells often tend to fold and shrink (Fig.4(a)).

Two major stress destabilized membrane properties during desiccation are fusion and lipid phase transitions (3). When water is removed, the packing of the polar head groups in the membranes

tightens, which leads to an increase in the phase transition temperature (3, 5, 23). This phase transition induces the lipids to reorganize, which leads to the destruction of the membrane-layer geometry. Lipid reorganization leads to leakage during desiccation and rehydration. This leakage is due to an increased membrane permeability in the dry cell, caused by a shift in membrane phospholipids from a lamellar phase at high water activities to a hexagonal phase at lower water activities (3, 12, 16).

Non-reducing disaccharides, such as sucrose and trehalose, can maintain membrane structures because these sugar molecules replace structural surface-bound water and also prevent phase transitions in the lipid bilayer (3, 5). However, we did not have successful results when using these disaccharides, for several possible reasons.

First, trehalose is effective only when the disaccharide can be taken up by the cells (4, 13). If sugar molecules can not enter the cell, these molecules can not prevent the changes in membrane structure due to metabolic unbalance and water loss. We selected DMSO as a possible solution to the penetration problem. Unfortunately, there was not much difference with or without DMSO.

Plant cells, unlike animal cells, have rigid cell walls; the plant cell is therefore altered by large hydrostatic pressures, important for the movement of water and solutes into and out of the cell. For usual physiological conditions, a positive hydrostatic pressure exists inside a plant cell. The existence of the internal hydrostatic pressure leads to stresses in its cell wall. By adjusting the solute concentration in an external solution, the internal

hydrostatic pressure can be reduced. The volume of the membrane-bounded body changes in response to variations in the osmotic pressure of the external solution. This is a consequence of the properties of membranes, which generally allow water to move readily across them, at the same time restricting the passage of certain solutes, such as trehalose. If the osmotic pressure of the external solution were increased even further, a greater amount of water would flow out of the cell (26).

Second, trehalose can be toxic to many plants (7, 11). ~~Configuration and steric factors are important in determining whether the action of a compound is toxic or protective to the cells (25).~~ We found that after keeping dinoflagellates in 1M trehalose for 2 days, most of the dinoflagellates died. However, this could be due to an osmotic pressure effect. ~~In the case of sucrose, if sucrose crystallizes, the hydroxyls will be unavailable, which causes mechanical disruption of the bilayer integrity.~~ Because of its tendency to crystallize, sucrose alone may not provide good membrane protection for desiccation (7, 11). Thus the choice of replacement additives is a critical factor in air drying.

Direct application of the classical methods which ~~had~~ been ~~carried out~~ with animal cells and tissues was generally unsatisfactory for dinoflagellates. ~~From these results~~, the degree of ~~protection during desiccation~~ and the proper additives depend on ~~many parameters~~, involving drying method and conditions, ~~membrane composition, and geometry.~~ Anhydrobiotic organisms must dry slowly in order to survive (20). We therefore tried a slow air drying process (Fig.3), which

produced more positive results (Fig.5). Slower drying may provide time for a metabolic transformation essential to survival (2). Dinoflagellates in 0.1M DMSO, 1% PEG and 5% PEG survived 3 days after drying; these were the best results.

~~We may explain these results in two ways.~~ ~~One,~~ bound water is necessary for the maintenance of their structural integrity (2, 17). DMSO and PEG solutions hold more water than the other solutions. DMSO, which penetrates the membrane and enters the cell, may prevent dehydration of the cell by maintaining ~~the~~ internal water pressure (4). Morris found the high molecular weight additive polyvinylpyrollidone(PVP, average ~~Mol.~~ wt.=40,000) did not induce a significant loss of water from cells before freezing (14). From his results, we expect ~~ed~~ to produce the same behavior with PEG, which has a high molecular weight. PEG also has an amphiphilic nature, which suggests that it might interact with cell membranes. PEG-membrane interactions are probably involved in membrane fusion (27). The effect of PEG depends both on the numbers of molecules bound per unit area of cell surface and on the binding energies characteristic of this interaction (27).

The other reason is the same as in the case of normal air drying. DMSO ~~produces~~ a pronounced increase in the phase transition temperature of phospholipid membranes, ~~indicating~~ an increased stability (24). But a high concentration of DMSO, such as 1M, was not ~~good~~, DMSO seemed to be toxic at such concentrations. ~~In the case of trehalose and sucrose, the results~~ were also not ~~good.~~

~~Figure 6 represents the cell morphologies after rehydration, following slow air drying. We see evidence of cell division, indicating the viability of these organisms after rehydration.~~ The morphology of the cells in 0.1M DMSO was not greatly altered (Fig.6[a]), ~~although~~ there was some shrinkage in the 0.1M DMSO mixed with 0.1M trehalose, presumably due to the effect of trehalose (Fig.6[b]).

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and evidence of cell division was observed.

cells exposed to

why?

~~CONCLUSIONS~~

We believe it is possible to air dry Pyrocystis lunula and related similar dinoflagellate. ~~We need~~ further research is needed in order to find the optimum drying rate, the best additives, and the optimum concentration of the additive for each specific organism. Such techniques could have application for food storage and medical supply preservation.

no underline

ACKNOWLEDGMENTS

We ~~would like to~~ thank M. Lisonbee, C. H. Ho and Z. W. Gu for helpful guidance. Special thanks to Protein Solutions, Inc. for partial support of this work.

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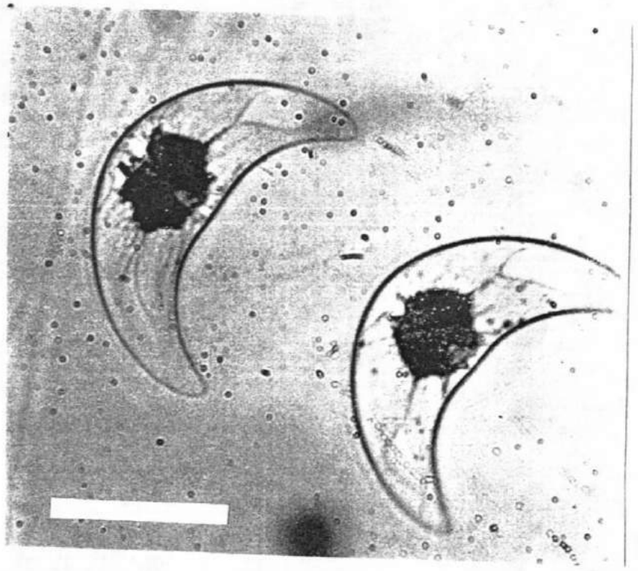


Fig. 1. Typical morphology of Dinoflagellate (Pyrocystis lunula). Bar: 0.1 mm.

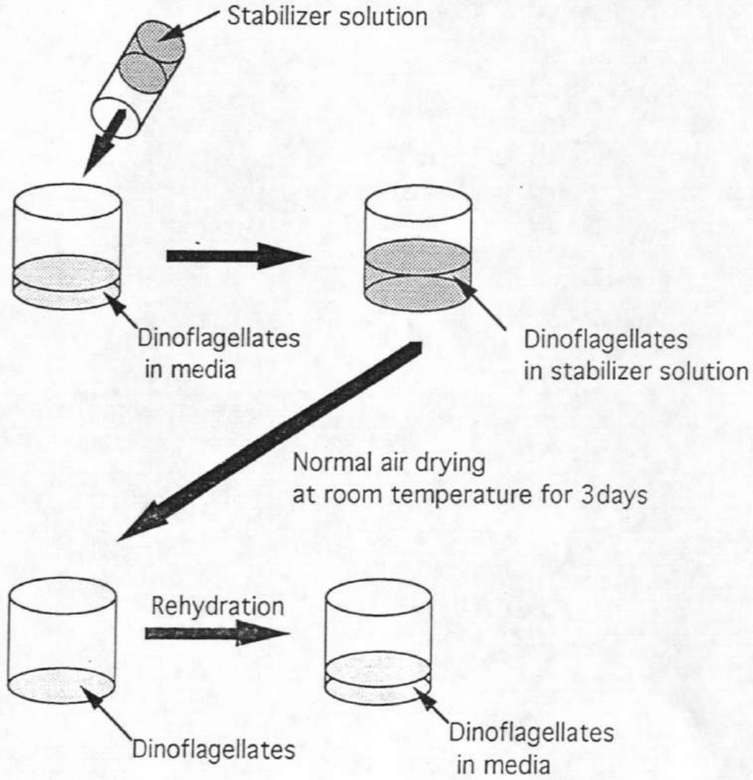


Fig. 2. The normal air drying process.

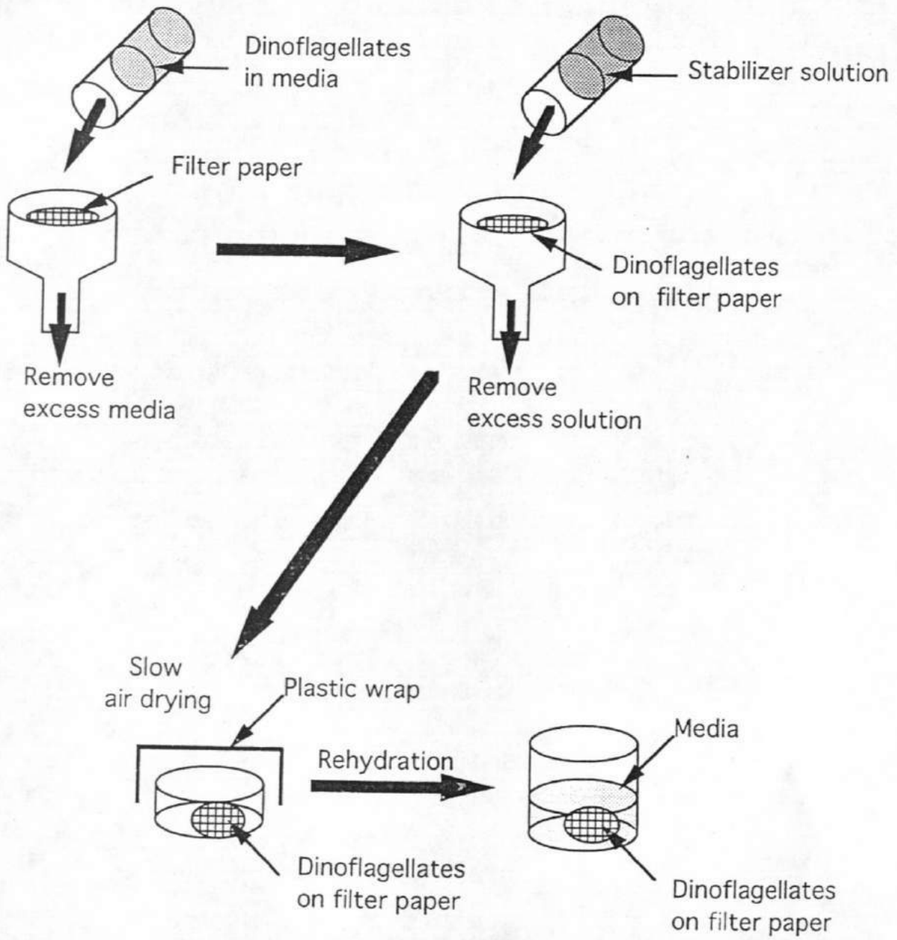
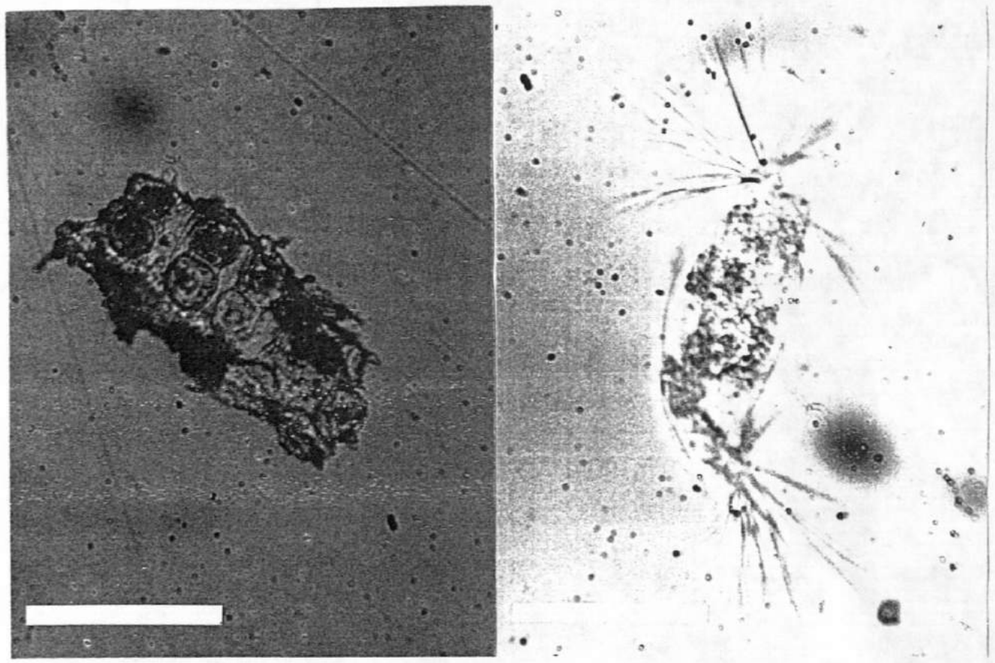


Fig. 3. The slow air drying process.



(a) (b)

Fig. 4. The morphologies during normal air drying after 2 days.
(a) In 0.1M trehalose: cell had shrunk with an altered, abnormal morphology.
(b) In 0.1M trehalose mixed with 0.1M DMSO: the cell membrane appears broken.
Bar: 0.1 mm.

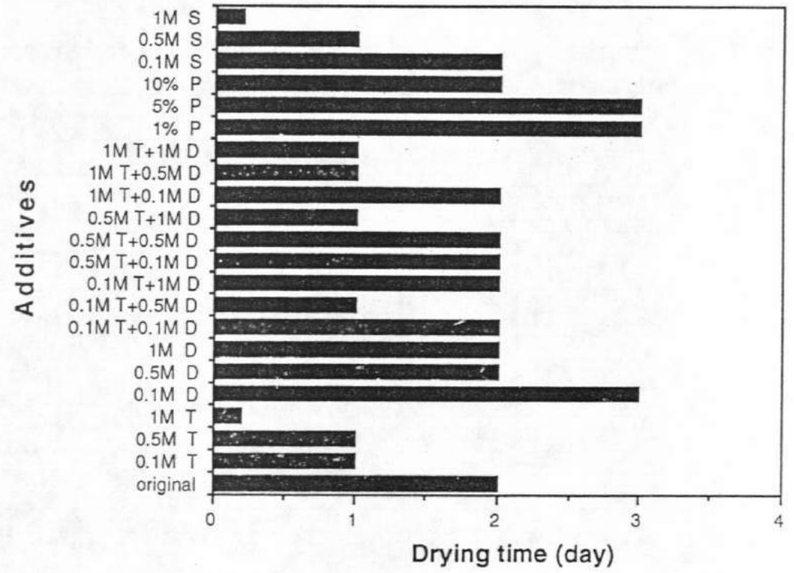


Fig. 5. The drying time from which Pyrocystis lunula recover after slow air drying.
S: sucrose; P: PEG; T: trehalose; D: DMSO

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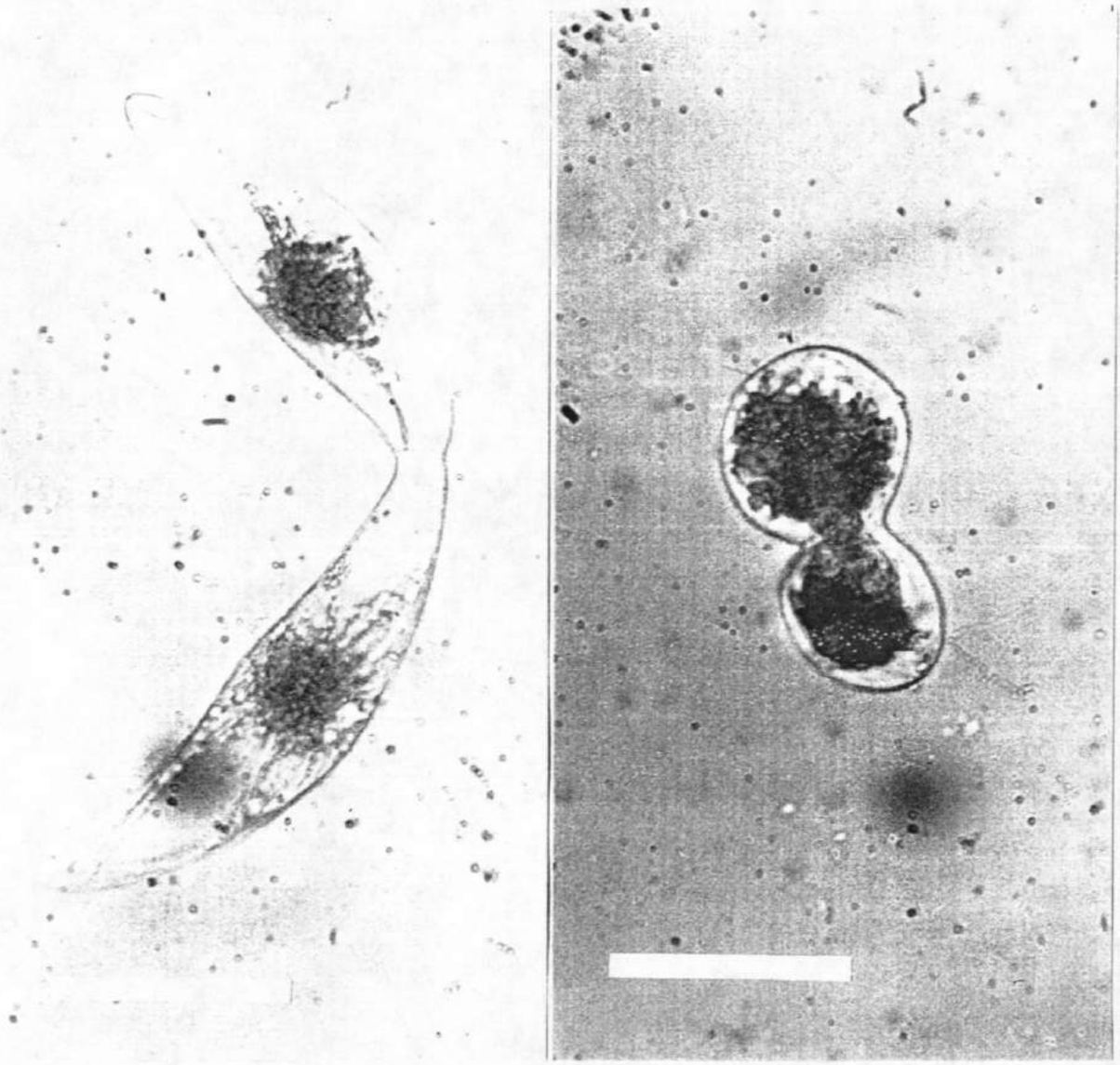

(a) (b)

Fig. 6. Pyrocystis lunula morphologies after rehydration after slow air drying for 2 days.
(a) 0.1M DMSO: apparent cell division.
(b) 0.1M trehalose mixed with 0.1M DMSO: apparent cell division with some shrinkage.
Bar: 0.1 mm

AIR/WATER MONOLAYER STUDIES OF BIOLUMINESCENT ENZYMES

D. J. Min[1], C. Y. Wang[2], and J. D. Andrade[2]
[1]Dept of Materials Science and Engineering and [2]Dept of Bioengineering, MEB 2480
University of Utah, Salt Lake City, Utah 84112, USA

Introduction

There is interest in the development of high-sensitivity enzymatic sensors for applications in medicine, biotechnology, and environmental science (1). Bioluminescent enzyme systems, such as bacterial and firefly luciferases, are among the most practical biosensors.

Immobilization of enzyme on solid supports is a key feature of many enzyme biosensors, allowing the use of minimum amounts of enzyme while providing for rapid response times.

The study of enzymes at interfaces is important for immobilization, because the films are often prepared by adsorption of enzyme onto the interface. The phenomenon of adsorption depends on the properties of the enzyme adsorbed and the nature of the substrate conditions (2, 3).

The air/water interface is a model hydrophobic surface for studying enzyme adsorption. The phenomenon of adsorption of some enzymes at this interface represents similarities to that at hydrophobic solid surfaces. Recently it has been of interest to use monolayers of enzyme at the air/water interface for the fabrication of ultrathin sensor membranes (4).

We discuss a preliminary study on the behavior of bacterial and firefly luciferases at the air/water interface with different aqueous phase conditions.

Materials and Methods

Bacteria (*V. fischeri*) and firefly (*Photinus pyralis*) luciferases were purchased from Sigma Chemical Co. Firefly luciferase was not purified further because it was pure as determined by SDS-PAGE. Bacteria luciferase was purified further by use of a Pharmacia 16/60 Superdex prep grade column and Mono Q HR 10/10 anion exchange column in a Pharmacia FPLC system. All other reagents were obtained from Sigma Chemical Co.

A Langmuir-Blodgett (LB) film balance (Sybron-Brinkmann, Germany) was used for monolayer experiments. The film balance trough was 15 cm wide, 70 cm long, and 0.6 cm deep, and the moving barrier speed was 0.02 cm/sec.

Injection of luciferase solutions (bacteria luciferase: 200 ul of 0.16 mg/ml and firefly luciferase: 50 ul of 1 mg/ml) was done by flowing the solution down a glass rod (0.5 cm diameter and 10 cm long) at room temperature (Fig. 1). We waited for 1hr before moving the barrier and compressing the surface film. While moving the barrier, the surface-area (π-A) isotherm was recorded by a computer connected to the LB film balance.

Tris buffer (pH 7.8) was used for enzyme solutions. Phosphate buffered saline (PBS, pH 7.4) was used as the aqueous phase, which was also adjusted to 1, 3, and 5M urea for various experiments. All chemicals purchased were used without any further purification.

Results and Discussion

The two-dimensional π-A isotherm is conceptually analogous to a three-dimensional pressure - volume (P-V) isotherm. Surface pressure is defined as the difference between the surface tensions of the clean and monolayer areas. When the area available to each molecule is much larger than the molecular dimension, the state of monolayer is "gas"-likes. As the surface area is reduced, the surface pressure become higher and the system progresses to a "liquid" and then "solid" phase.

Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects
A. K. Campbell, L. J. Kricka and P. E. Stanley, eds., John Wiley & Sons
(New York, 1994) pp. 494-497.

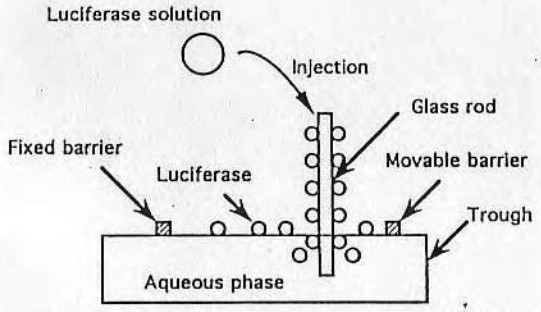

Figure 1. Experiment of π-A isotherm at the air/water interface.

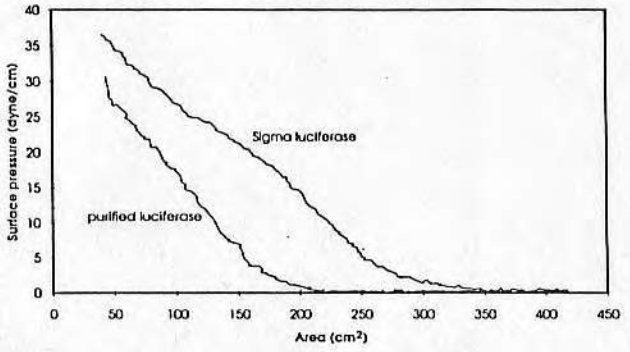

Figure 2. The π-A isotherms of as-received Sigma and purified bacteria luciferase at the air/water interface. 200 ul (0.16 mg/ml) was injected onto the interface. Aqueous phase is PBS.

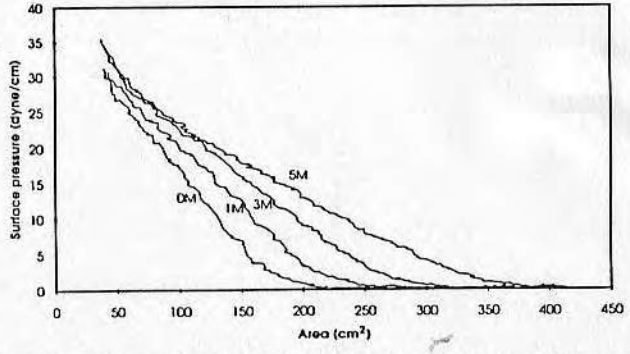

Figure 3. The π-A isotherms of purified bacterial luciferase as a function of urea concentrations (0, 1, and 5M) in the aqueous phase. 200 ul (0.16 mg/ml) was injected onto the interface.

Enzymes at the interface change their conformations to reduce the interfacial energy, driven by hydrophobic interactions, which lead to unfolding. The degree of enzyme conformational change depends on the properties of the enzyme adsorbed, interfacial energy, and the residence time (5).

Figure 2 shows π-A isotherms of as-received Sigma and purified bacteria luciferase at the air/water interface: surface pressure of as-received material rises at larger surface area than does the purified material. The as-received Sigma product is 50 % purity - it is clear that the many other proteins present are very surface active and dominate the π-A behavior.

Figure 3 shows π-A isotherms of purified bacteria luciferase as a function of different urea concentration of substrate: bacteria luciferase apparently unfolds rapidly when it contacts the high urea concentration of the substrate solution. Substrate condition is very important for the change of enzyme structure at the air/water interface because the hydrophobic interaction at this interface depends mainly on water conditions. Water structure and enzyme molecules themselves are influenced by solute concentration and pH in aqueous phase. Urea disrupt the usual aqueous hydrogen-bond network by hydrogen bonding with water; the surface tension of aqueous phase increase with increasing urea concentration (6). Urea is a common used protein denaturant; protein unfolding at the air/water interface is accelerated in the presence of high urea concentrations.

Figure 4 shows π-A isotherms of firefly luciferase according to different urea concentration in the substrate solution: behavior of this luciferase at the air/water interface is almost the same, regardless of urea concentration. From this figure, we recognize that firefly luciferase is largely unfolded at the air/water interface, in contrast to bacterial luciferase.

In summary, bacteria luciferase is "hard" protein and firefly luciferase is "soft" protein, using the terminology of Norde, et al (7, 8), even though they have similar thermal denaturation temperatures.

We need to further investigate the propeties of both luciferases. Some properties of both enzymes are shown in Table 1. The relationships between luciferases and solution conditions must be studied in order to further understand the behavior of luciferases at interfaces and thus optimize luciferase activity in biosensors.

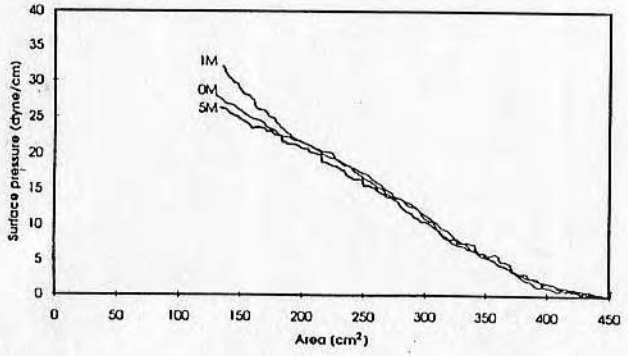

Figure 4. The π-A isotherms of firefly luciferase as a function of different urea concentrations (0, 1, 3, and 5M) in the aqueous phase. 50 ul (1 mg/ml) was injected onto the interface.

Table 1. Properties of bacterial and firefly luciferases

	Bacteria luciferase	Firefly luciferase
Structure	Heterodimer	Monomer
Molecular weight	76 kD	62kD
Isoelectric point	*	6.2-6.5
Denaturation temperature	40 C	44 C
Hydrophobic residues (%)	*	60
Number of disulfide bond	0	0

* sequence analysis in process.

Acknowledgements

We thank Prof. J. N. Herron for access to the Pharmacia FPLC facilities.

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PRELIMINARY STUDY OF THE OPTIMUM CONDITIONS FOR A LACTATE SENSOR BASED ON BACTERIAL BIOLUMINESCENCE

DJ Min and JD Andrade
Dept. of Materials Science and Engineering, University of Utah,
Salt Lake City, Utah 84112, USA

Introduction

The bacterial bioluminescence system can be applied as an enzyme sensor for biochemicals related to reduced nicotinamide adenine dinucleotide (NADH). A key problem is completeness of the conversion of reactants to products, because the efficiency of conversion affects the sensitivity. This problem is especially significant in multiple enzyme systems since each enzyme may have different optimal conditions. The selection of the optimal pH value, the effect of reactants and products on the enzyme activities, and the enzyme concentrations and their ratios are important in the optimization of enzyme sensors (1).

A lactate sensor was chosen as a model system because lactate is important in clinical analysis, food analysis, and sports medicine (2). Lactate monitoring using bacterial bioluminescence has many advantages, including simplicity and speed. The governing reactions are:

$$\text{lactate} + NAD^+ \xleftarrow{\text{LDH}} \text{pyruvate} + NADH + H^+ \quad (1)$$

$$NADH + FMN + H^+ \xrightarrow{\text{OR}} NAD^+ + FMNH_2 \quad (2)$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{LF}} FMN + RCOOH + H_2O + \text{light} \quad (3)$$

where NAD is ß-nicotinamide adenine dinucleotide, LDH is lactate dehydrogenase, FMN is flavin mononucleotide, OR is NADH:FMN oxidoreductase, $FMNH_2$ is reduced flavin mononucleotide, RCHO is decanal, and LF is bacterial luciferase. NADH formation is catalyzed by LDH. Light is emitted after the serial reactions by OR and LF. The light intensity is proportional to the rate of NADH formed, which is proportional to lactate concentration in the solution.

We will discuss optimal conditions for lactate analysis by bacterial bioluminescence and the interference reactants and products on the reactions.

Materials and Methods

Instrumentation: Bioluminescence was measured using a photon counting spectrofluorometer (I.S.S. Inc., Champaign, Ill., USA). The unit of light intensity is relative light units (RLU).

Reagents: All assays were performed in 500 mL solution of 0.1 mol/L phosphate buffer at room temperature. The final concentrations of NAD (Calbiochem, La Jolla, USA), lactic acid (Sigma, St. Louis, USA), FMN (Boehringer Mannheim, Indianapolis, USA), and decanal (Sigma, St. Louis, USA) in the assay are 0.25 mmol/L, 1 mmol/L, 10 µmol/L, and 0.001 %, respectively. In the interference experiments, the final concentrations of NAD, lactic acid, and pyruvate (Sigma, St. Louis, USA) were changed to the desired concentrations and the final concentration of NADH (Sigma, St. Louis, USA) was 0.1 mmol/L. The concentrations of stock solutions for LDH (Calbiochem, La Jolla, USA), LF (Sigma, St. Louis, USA), LF (Boehringer Mannheim, Indianapolis, USA), and OR (Boehringer Mannheim, Indianapolis, USA) were 2000 U/mL, 6 mg/mL, 7.5 mU/mL, and 5 U/mL, respectively. The amounts of the three enzymes were changed in each experiment. All reagents and enzymes were used without further purification.

Procedures: All assays were performed by adding reagent mixtures to the cuvettes containing LF, OR, and/or LDH in the different combinations. To test the interference in the reactions, the pH of reaction mixtures was titrated to 7.0 and 50 µL of Sigma LF stock solution was applied. Since Sigma LF already contained OR as a minor contaminant, it was not necessary to add OR. To obtain the optimal pH conditions, 50 µL each of Sigma LF and LDH stock solutions were added. For determining the optimal amounts of enzymes, the pH of reaction mixtures was adjusted to 7.6 and stock solutions of Boehringer LF, OR, and LDH were used.

Results and Discussion

Light intensity is dependent on the rates of the reactions in the bioluminescent system (3). The higher concentrations of reactants and/or higher activity of enzymes give higher light infensity. At a given concentrations of reactants, the light intensity depends solely on the enzyme activities. The effects of reactants and products in the serial lactate reactions were tested. Fig. 1 shows the effects of lactate, NAD, and pyruvate on the bioluminescent system. Although there was slight inhibition, it was not critical to the bioluminescence activity within the tested concentration range. Prahl et al. (4) found that a large excess of lactate, NAD, and pyruvate inhibit LDH activity, and an excess of NAD also inhibits LF activity. NAD concentration in their experiments was higher than our test range. The purity of reagents seemed to be important for these tests. The slight increase of light intensity at higher concentration of NAD and lactate was probably due to the impurity of NADH.

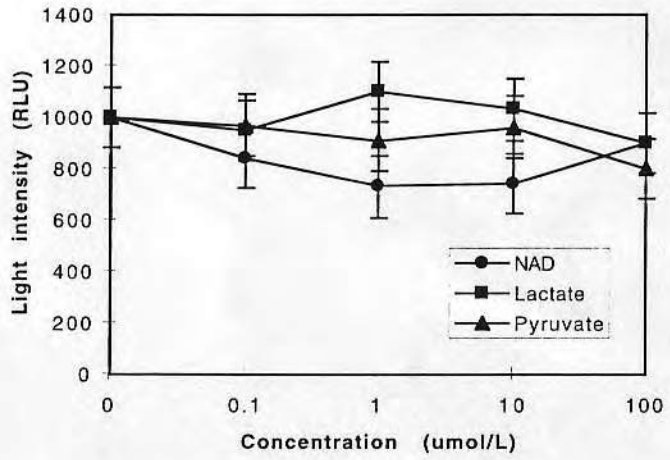

Figure 1. The effects of lactate, NAD, and pyruvate on the activity of the bioluminescent reactions. The reaction mixtures (pH 7.0) contain 0.1 mmol/L NADH, 10 µmol/L FMN, 0.001 % decanal, and 50 µL Sigma LF (6 mg/mL).

The optimum pH of bacterial bioluminescent reactions is known to be 7.0 and the reaction is inhibited at pH above 8.0 (5). However, the optimal pH of LDH reaction in the direction of NADH formation is 9.0-9.6 (6). The consumption of newly formed NADH in the LDH reaction by the other bioluminescent reactions allows sub-optimal pH conditions to be used, so the sensor can operate at pH below 8.0. An optimum pH of 7.6 was determined for the lactate sensor (Fig. 2). At pH below 7.6, NADH formation is likely inhibited; bioluminescent reactions may be inhibited at pH above 7.6. Prahl et al. (4) chose

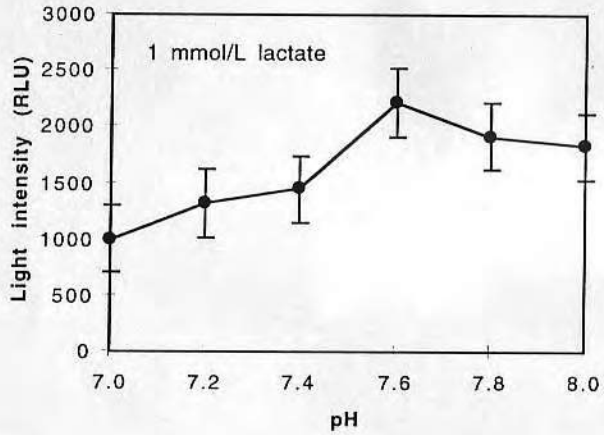

Figure 2. Light output as a function of pH in the lactate sensor. The reaction mixtures contain 1 mmol/L lactate, 0.25 mmol/L NAD, 10 µmol/L FMN, 0.001 % decanal, 50 µL (100 U) LDH, and 50 µL Sigma LF (6 mg/mL).

a pH of 7.4 for these reactions.

Low enzyme amounts result in a small dynamic range. Very high enzyme amounts are not economical for measuring low reactant concentrations. An optimum amount of enzyme for a given measurement range is important. Fig. 3 shows the effect of enzyme amount on light intensity. With 15 µL (75 mU) OR and 50 µL (100 U) LDH, saturation of light output occurred above 35 µL (0.26 mU) LF for 1 mmol/L lactate. The OR and LDH amounts used were experimentally determined from a set of optimizing experiments using 1

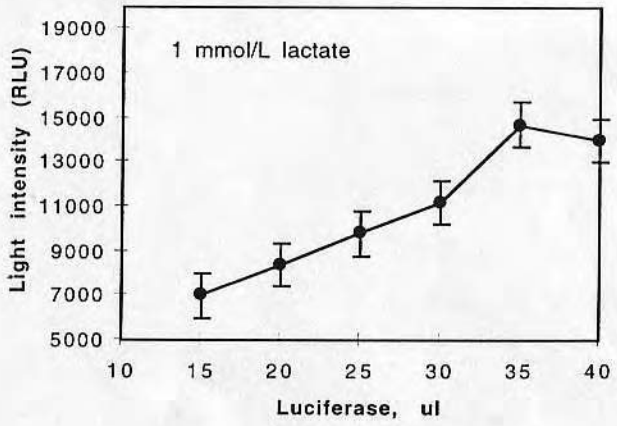

Figure 3. The effects of enzyme amounts on the light intensity in the bioluminescent lactate sensor. The reaction mixtures (pH 7.6) contain 1 mmol/L lactate, 0.25 mmol/L NAD, 10 µmol/L FMN, 0.001 % decanal, 50 µL (100 U) LDH, and 15 µL (75 mU) OR.

mmol/L lactate. With 75 mU OR and 0.26 mU LF, the light intensity did not change until 10 U LDH. Thus, the optimal ratio of LDH, OR, and LF was 10 U:75 mU:0.26 mU for 1 mmol/L lactate.

In summary, the inhibition effects of lactate, NAD, and pyruvate on the bioluminescent reactions were not critical. The optimum conditions for a lactate sensor based on bacterial bioluminescence (1 mmol/L lactate) were 10 U LDH, 75 mU OR, and 0.26 mU LF at pH 7.6.

This study is useful for the design of bioluminescent lactate sensors having different substrate ranges. The optimum enzyme amounts may depend on the lactate concentration to be measured.

Acknowledgements

We thank Prof. J. N. Herron for access to the photon counting spectrofluorometer. This work is partially supported by Protein Solutions, Inc., Salt Lake City, Utah via an STTR grant from the US National Science Foundation.

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Behavior of Model Proteins, Pretreated in Urea and/or Dithiothreitol, at Air/Solution Interfaces

Dong J. Min,* Lynn Winterton,† and Joseph D. Andrade*,[1]

**Department of Materials Science and Engineering, University of Utah, Salt Lake City, Utah, 84112; and*
†CIBA Vision Corporation, 11460 Johns Creek Parkway, Duluth, Georgia, 30136

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The behavior of the model proteins, lysozyme, myoglobin, and β-casein, pretreated in urea and/or dithiothreitol, at air/solution interfaces was studied by surface pressure–area techniques. The data suggested that in the absence of pretreatments the globular proteins are only partially unfolded at the interfaces. The interfacial activity was enhanced by the pretreatment (lysozyme in 8 M urea with 0.2 M dithiothreitol and myoglobin in 8 M urea). The interfacial activity of casein, a random-coil type protein, was not influenced by the pretreatment (8 M urea), as it readily and completely unfolds at the interfaces. The unfolding of globular proteins at the interfaces is apparently restricted by both disulfide and noncovalent bonds. Pretreatment can relax those restrictions, resulting in more complete interfacial unfolding.

Key Words: **lysozyme; myoglobin; casein; pressure–area; monolayer; urea; dithiothreitol.**

INTRODUCTION

The behavior of proteins at air/fluid interfaces have been studied for many years (1, 2). Protein monolayers at interfaces have received much attention in the food and pharmacology industries (3, 4). Study of protein monolayers is also important for understanding the native structure and stability of proteins (5).

Proteins at air/fluid interfaces are usually denatured or unfolded because interfacial interactions are generally stronger than the cohesive interactions within the protein molecule (6). The degree of unfolding and time taken to unfold at the interface depend on protein structure and on its polar–nonpolar character, which are determined by the amino acid composition: proteins with a relatively higher nonpolar character and a relatively random structure tend to experience higher degrees of unfolding at air/water interfaces, producing the denatured state more quickly than proteins with more polar character (7, 8).

Many studies have dealt with the role of structure in the unfolding of protein at interfaces. Evans *et al.* (9) suggested that proteins with only weak forces maintaining the tertiary structure have a higher probability of unfolding. Krebs *et al.* (10) found that the steady state surface activity depends on the content of nonpolar residues, without regard for the details of the secondary and tertiary structures. Norde *et al.* (11) discussed the structural rearrangements of a protein at the interfaces in terms of its structural stability in solution, its "softness" or "hardness"; a soft protein rearranges or denatures more easily than a hard protein. Andrade *et al.* (5) also examined various parameters correlating the behavior of proteins at interfaces and in solution, including denaturation temperature, number of disulfide bonds, molecular weight, and percent of nonpolar residues.

Many different types of interactions, including hydrogen bonding, electrostatic bonding, disulfide bonds, hydrophobic bonding, and dispersion forces, contribute to protein stability (12, 13). The rearrangement or unfolding of proteins at interfaces can be accelerated by removing the covalent and/or noncovalent bonds which restrict conformational changes. Urea, a denaturing agent, and dithiothreitol (DTT), a reducing agent, tend to accelerate the unfolding of proteins at interfaces by disrupting most of the noncovalent bonds and reducing the disulfide bonds, respectively (14, 15).

In this paper the interfacial behaviors of three model proteins, lysozyme, myoglobin, and β-casein, at the air/solution interface, as a function of urea and/or DTT pretreatments of the proteins, are reported and discussed in relation to their characteristics.

MATERIALS AND METHODS

Materials

Lysozyme (hen egg white, M = 14 kDa, Boehringer Mannheim), myoglobin (horse heart, M = 18 kDa, Sigma), and β-casein (bovine milk, M = 24 kDa, Sigma) were used without any further purification.

Phosphate buffer (10 mM PBS, pH 7.4) was made from phosphate-buffered saline tablets (Sigma). Urea (Mallinck-

[1] To whom correspondence should be addressed. E-mail: joe.andrade@m.cc.utah.edu.

0021-9797/98 $25.00

rodt) and DTT (Aldrich) were also used without any further purification.

Methods

PBS buffer was used for all protein solutions and subphase solutions. Protein solution concentrations were measured by UV absorbance at 280 nm. Protein solutions were also prepared containing urea and/or DTT.

The Langmuir–Blodgett (LB) balance (SYBRON-Brinkmann, Germany) used consists of a trough 15 cm wide, 70 cm long, and 0.6 cm deep, with a barrier speed of 0.02 cm/s. The various protein solutions (200 μl of 1 mg/ml lysozyme, 200 μl of 1 mg/ml myoglobin, and 200 μl of 0.1 mg/ml β-casein) were allowed to flow down a glass rod (0.5 cm diameter and 10 cm long) at room temperature. The protein solution was layered and spread along the air/solution interface; some entered into the subphase. One hour or more was allowed to reach a "steady-state" of the protein state at the interface before moving the barrier and compressing the surface film. The surface pressure–area isotherms were obtained via computer control of the LB balance. Area is the cross-sectional area of the trough.

RESULTS AND DISCUSSION

Globular proteins of amphiphilic character unfold at the air/solution interface, driven mainly by the hydrophobic interactions (3, 6, 16). Depending on its conformational stability (its "hardness" or "softness"), the protein conformationally adapts at the interface to minimize the interfacial tension (11, 16). Such conformational adaptation is a strong function of the structural parameters, such as molecular size, hydrophobicity, flexibility, and charge (17).

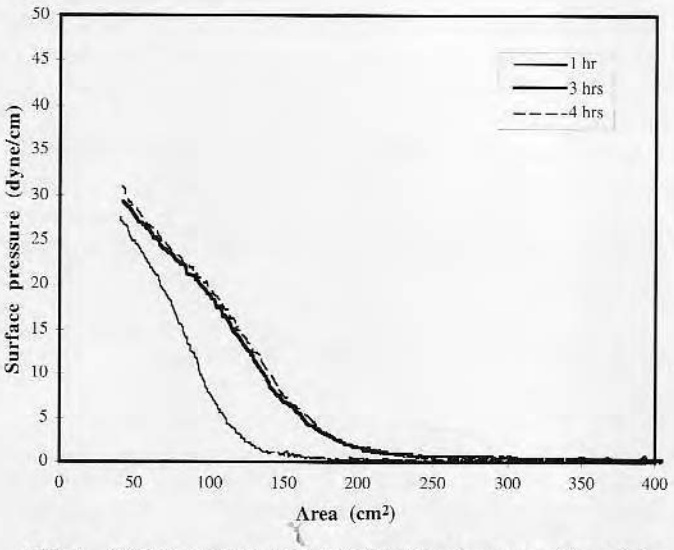

FIG. 1. Surface pressure-area plots for native lysozyme. The solution (200 μl of 1 mg/ml) was spread onto the interface. Lysozyme at the interface reached a "steady-state" after 3 hours. The solution and the subphase were PBS.

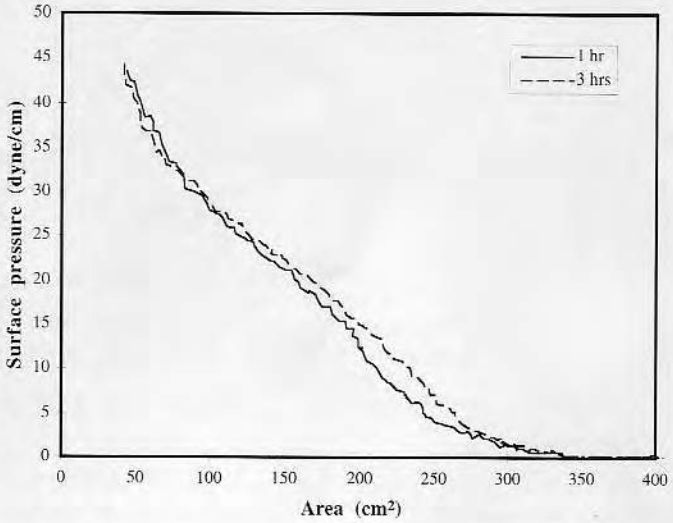

FIG. 2. Surface pressure-area plots for native myoglobin. The solution (200 μl of 1 mg/ml) was spread onto the interface. Myoglobin at the interface reached a "steady-state" within 1 hour. The solution and the subphase were PBS.

Lysozyme and myoglobin were selected as model globular proteins because they have similar dimensions but have different structural and stability properties. Myoglobin is "softer" than lysozyme. As expected, myoglobin shows higher interfacial activity than lysozyme; the time to reach "steady-state" of myoglobin at the interface was shorter and the area occupied by myoglobin was higher than that for lysozyme at the same surface pressure (Fig. 1 and 2). Lysozyme at the interface was only slightly unfolded, even after a long time at the interface, and reached an apparent "steady-state" after 3 h, probably because it is relatively stable due to the disulfide bonds (Fig. 1). "Time" means the elapsed time between spreading and the start of compression. Myoglobin achieved an apparent "steady-state" within 1 h, probably due to its higher hydrophobicity and flexibility (Fig. 2). In general, the results obtained agree with those reported in the literature (9, 18).

The effect of electrostatic interactions between protein molecules at the interface should also be considered because both proteins have different isoelectric points (pI values of lysozyme and myoglobin are 10.7 and 7.8, respectively). Spreading of protein molecules on the interface leads to a monolayer in which the proteins are oriented as well as conformationally changed. The configuration of the proteins in the monolayer is expected to be different from the native state in solution. Graham *et al.* (28) found that the adsorption of lysozyme on a hydrophobic interface, like the air/water interface, is insensitive to pH in the range 1–12 although adsorption on a hydrophilic surface is maximal at the pI, presumably due to electrostatic repulsion. Their work on

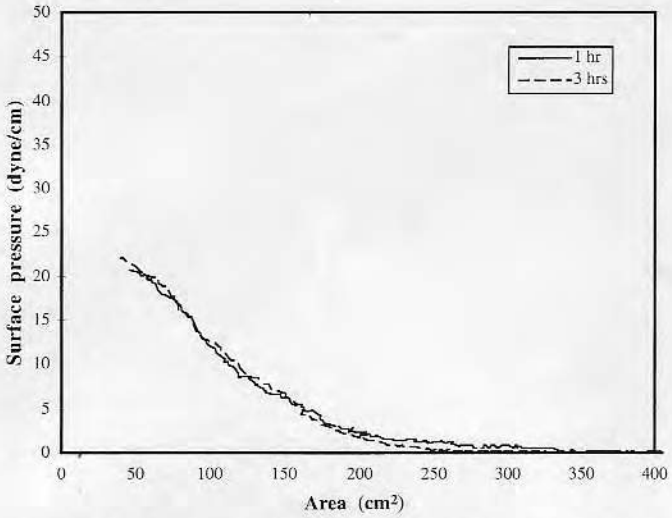

FIG. 3. Surface pressure-area plots for native β-casein. The solution (200 μl of 0.1 mg/ml) was spread onto the interface. Casein at the interface reached a "steady-state" within 1 hour. The solution and the subphase were PBS.

lysozyme shows that the protein behavior on the air/water interface is dominated by hydrophobic interactions. Thus the electrostatic contributions on protein behavior at the air/solution interface is neglected here.

Assuming a close-packed monolayer and a cross-sectional area for both proteins of 9×10^{-14} cm^2 (lysozyme, 45 $\times$ 30 $\times$ 30 Å (5); myoglobin, 45 $\times$ 35 $\times$ 25 Å (29)), the increase of the surface pressure for a given protein concentration (200 μl of 1 mg/ml) is expected to be about 700 cm^2. Since the cross-sectional areas of protein at the steady-state are increased, likely due to the unfolding, the area occupied by a protein molecule expands, meaning the surface pressure plot should increase above 700 cm^2. The results show that the surface pressure does not increase until the area is decreased to about 250 and 300 cm^2 for the isotherms of lysozyme (3 h) and myoglobin (1 h), respectively, after steady-state was achieved. Our results suggest that many of the globular proteins at the interface diffuse into the subphase.

β-Casein was selected as a model random-coil type protein. Because of the high molecular size of β-casein and the limited size of the trough's area (1050 cm^2) a spread solution concentration of 0.1 mg/ml was used. Casein shows much higher interfacial activity than the globular proteins (Fig. 3). Assuming a close-packed monolayer and a cross-sectional area of 6.6×10^{-13} cm^2 (radius of gyration = 46 Å (25)), the surface pressure should increase at about 330 cm^2, which is what our data shows. Since the dimension of "unfolded" random-coil protein may be similar to the native form, most of the β-casein remained at the interface.

The surface pressure–area isotherms depend on the number and the dimension of molecules at the interface and thus relate to the conformational adaptation of proteins. Protein behavior at the air/solution interface depends on protein characteristics. Conformational adaptation is a strong function of protein structure. The interfacial activity of proteins can be enhanced by decreasing the covalent and noncovalent bonds which restrict conformational changes. Urea tends to produce a randomly coiled state by disrupting most of the noncovalent interactions which stabilize protein structure (14, 19, 20). Disulfide bonds in lysozyme are reduced and removed by use of another denaturant, DTT.

Lysozyme pretreated in 8 M urea shows almost the same surface pressure–area isotherms as native lysozyme (Fig. 4): the time effect and the degree of unfolding are the same. Lysozyme pretreated in 0.2 M DTT shows no significant time effect at low surface pressure resulting in the same unfolding state as native lysozyme (Fig. 5). Lysozyme pretreated in 8 M urea with 0.2 M DTT shows no time effect but a much higher degree of unfolding (Fig. 6). Greater unfolding of myoglobin is achieved by pretreatment in 8 M urea (Fig. 7). Myoglobin does not have disulfide bonds, so a DTT effect was not expected.

Thus unfolding of globular proteins at the air/solution interface, driven by hydrophobic interactions, is accelerated by the disruption of noncovalent bonds. If the protein has disulfide bonds, the disulfide bond is indeed very important in the interfacial behavior of protein, including time effects and degree of unfolding. The interfacial activity of such proteins can be accelerated by decreasing both disulfide and noncovalent bonds.

Lysozyme did not exhibit much change at the interface due to pretreatment in 8 M urea or 0.2 M DTT. Pike *et al.* (21) found that urea does not significantly affect the

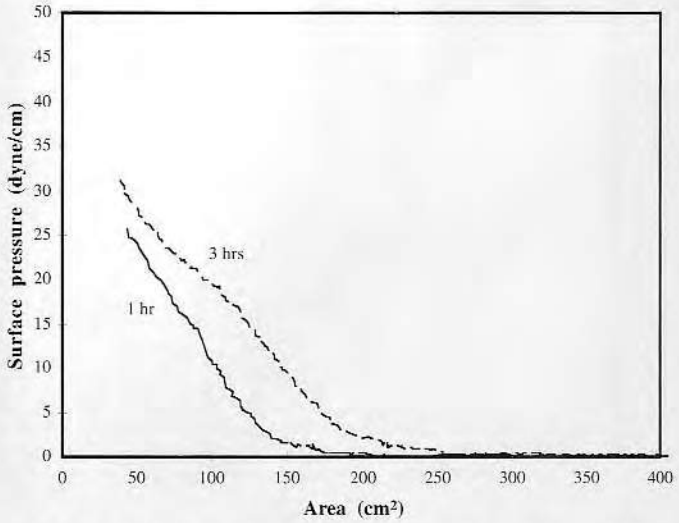

FIG. 4. Surface pressure-area plots for lysozyme pretreated in 8M urea. The solution (200 μl of 1 mg/ml) was spread onto the interface. The subphase was PBS. These results are almost the same as for native lysozyme: the time effect and the degree of unfolding were the same.

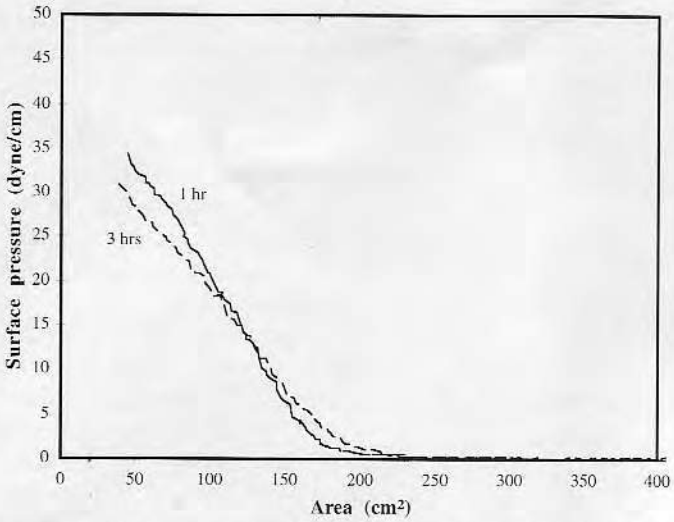

FIG. 5. Surface pressure-area plots for lysozyme pretreated in 0.2M DTT. The solution (200 μl of 1 mg/ml) was spread onto the interface. The subphase was PBS. Lysozyme at the interface reached a "steady-state" within 1 hour, but the degree of unfolding was not significantly different from native lysozyme.

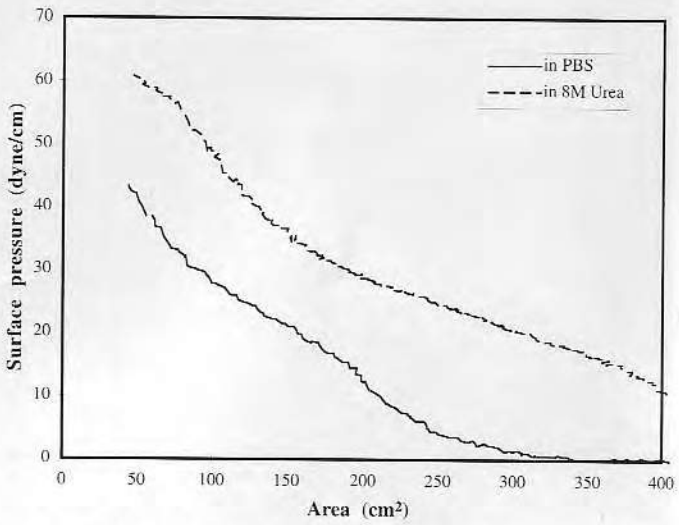

FIG. 7. Surface pressure-area plots for myoglobin pretreated in 8M urea. The solution (200 μl of 1 mg/ml) was spread onto the interface. The subphase was PBS. The degree of unfolding is higher than for native myoglobin.

conformation of the crystal structure of lysozyme, even at high concentrations. Pace (20) also found that urea denaturation does not always lead to complete unfolding. Even though most of the noncovalent bonds are broken by urea, conformational changes are restricted by the disulfide bonds. The literature claims that reduction of disulfide bonds in proteins results in considerable changes in the configuration of the polypeptide chain (14, 22). However, such conformational changes produced by reduction of the disulfide bonds depend on the number and location of the disulfide bonds broken. Sometimes DTT cannot reduce all the disulfide bonds in proteins because some are buried in the structure (23). Many groups (2, 12, 24) think that the unfolding rate at the interface is mainly dominated by the disulfide bonds.

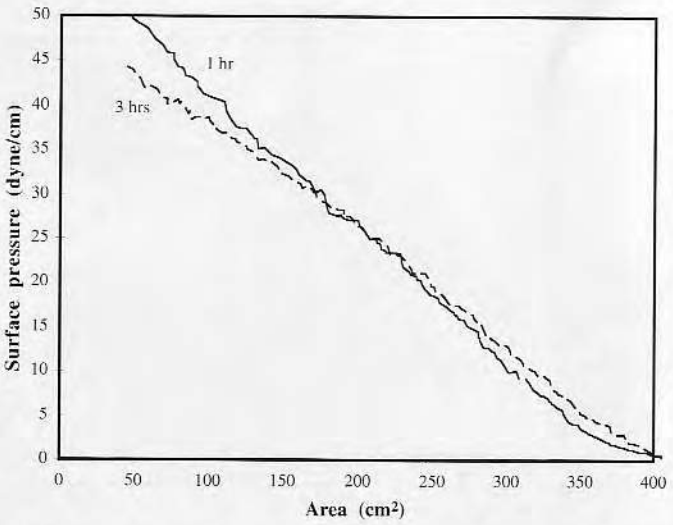

FIG. 6. Surface pressure-area plots for lysozyme pretreated in 8M urea with 0.2M DTT. The solution (200 μl of 1 mg/ml) was spread onto the interface. The subphase was PBS. Lysozyme at the interface reached a "steady-state" within 1 hour, and the degree of unfolding is much higher than for the other cases.

Lysozyme in 8 M urea may have some noncovalent bonds disrupted, but all of the disulfide bonds are retained; the disulfide bonds restrict the conformational change, thus the time effect and the degree of unfolding are not changed. Lysozyme in 0.2 M DTT may have some disulfide bonds reduced but retains most of its noncovalent bonds: although the reduced disulfide bonds make it easier for conformational changes to occur, the unfolding is restricted by the noncovalant bonds and thus there is little time. However, lysozyme in 8 M urea with 0.2 M DTT exhibits more unfolding than in PBS, meaning most of the noncovalent and disulfide bonds are broken. We hypothesize that the conformational changes induced by urea facilitate the interactions between DTT and the disulfide bonds, and the disulfide bonds reduced facilitate interactions between urea and noncovalant bonds.

The interfacial activity of β-casein was not influenced by pretreatment with 8 M urea, because this protein has a flexible, random-coil structure under normal conditions (Fig. 8). Although the structure of β-casein is not truly random (25, 26), the interfacial activity is not greatly influenced by the pretreatment. β-Casein was already unfolded at the interface without pretreatment (1, 9).

Thus, the globular proteins do not totally unfold at the interface. This result was also obtained by Boyd *et al.* (27) who found that globular proteins give interface films which have a higher interfacial viscosity than those formed by random-coil proteins. The increase of interfacial area by pre-

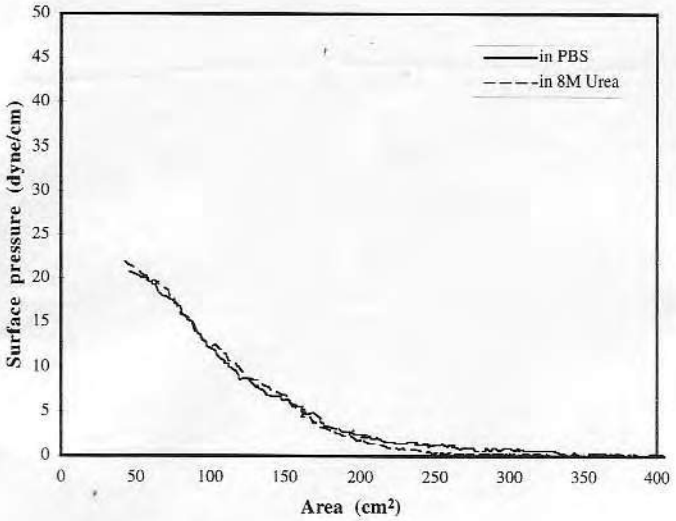

FIG. 8. Surface pressure-area plots for β-casein pretreated in 8M urea. The solution (200 μl of 0.1 mg/ml) was spread onto the interface. The subphase was PBS. The degree of unfolding was not significantly different from native casein.

treatment relates to the increasing unfolding. The loss of protein into the subphase was also decreased with increase in unfolding: more flexible globular proteins, when pretreated, have a greater chance of unfolding before loss.

SUMMARY

Protein behavior at the air/solution interface has been studied by pretreatment of protein before spreading. For the globular proteins, lysozyme and myoglobin, unfolding at the interface was accelerated by the pretreatments, indicating that the native globular proteins do not totally unfold at the interface. Lysozyme in 8 M urea with 0.2 M DTT and myoglobin in 8 M urea exhibit a higher degree of unfolding at the interface than native protein. The unfolding of the random-coil type protein, β-casein, at the interface is not influenced by urea, as it is already completely unfolded at the air/solution interface.

Thus unfolding of globular proteins at the air/solution interface is restricted by both disulfide and noncovalent bonds. The unfolding rate of lysozyme is mainly influenced by its disulfide bonds. The increase of the interfacial area as a result of the pretreatment relates to increasing unfolding and decreasing loss into the subphase.

ACKNOWLEDGMENTS

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DEVELOPING A BIOSENSOR FOR L-PHENYLALANINE BASED ON BACTERIAL BIOLUMINESCENCE

DJ Min[1], RJ Stewart[2], and JD Andrade[2]
[1]Dept. of Materials Science and Engineering, and [2]Dept. of Bioengineering, University of Utah, Salt Lake City, UT 84112, USA

Introduction

The determination of L-phenylalanine (Phe), an essential amino acid for mammals, is of great interest in several fields, including medicine, food science, and biotechnology (1). The measurement of Phe levels is important in screening for phenylketonuria (PKU), an inborn error of metabolism, and for monitoring the dietary management of PKU patients (2). Most Phe measurements, including microbial, fluorimetric, enzymatic, and chromatographic assays, are performed in centralized laboratories, often resulting in delays, inconvenience, and significant costs (2)

There is growing interest in self-monitoring and self-treatment of PKU in the home environment. Self-monitoring of the blood Phe concentration is important so patients can manage their diets and maintain appropriate Phe levels. There have been efforts at developing easy-to use assays for Phe (3).

Bacterial bioluminescence is a powerful and sensitive analytical tool. A flow sensor for Phe using immobilized phenylalanine dehydrogenase (PheDH), FMN:NAD(P)H oxidoreductase (OR), and bacterial luciferase (LF) was developed based on the following reactions (4);

$$\text{Phe} + \text{NAD}^+ + \text{H}_2\text{O} \xleftarrow{\text{PheDH}} \text{phenylpyruvate} + \text{NH}_3 + \text{NADH} + \text{H}^+ \quad 1)$$

$$\text{NADH} + \text{FMN} + \text{H}^+ \xrightarrow{\text{OR}} \text{NAD}^+ + \text{FMNH}_2 \quad 2)$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{LF}} \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light} \quad 3)$$

where NAD is nicotinamide adenine dinucleotide, NADH is the reduced form of NAD, FMN is flavin mononucleotide, $FMNH_2$ is the reduced form of FMN, and RCHO is long chain aldehyde. However, this method is not suited for self-monitoring of Phe, because it involves a peristaltic pump, preparation of solution, and a sample injection system.

A dip-stick type sensor is needed for self-monitoring of Phe in the home environment. Dip-stick devices are, in principle, easy to design and manufacture, resulting in reduced cost. In addition, they are easy to handle and use by people without special techniques or training.

We are developing an inexpensive dip-stick type-Phe sensor based on bacterial bioluminescence for self-monitoring in the home environment. This study evaluated the feasibility of a high performance Phe sensor based on the PheDH, OR, and LF reactions, and its optimal reaction conditions in homogeneous solution.

Materials and Methods

OR and LF were produced from E. coli by using recombinant techeniques. Their purity were over 90 % and their specific activity were close to the enzymes from Boehringer. PheDH (Cat. No. P-4798) was purchased from Sigma (St. Lousis, MO, USA) and used without further purification.

NADH (Cat. No. 128023) and FMN (Cat. No. 476501)were purchased from Boehringer (Indianapolis, IN, USA). Dodecanal (Cat. No. D-3042), Phe (Cat. No. P-8324), and phenylpyruvic acid (Cat. No. P-8126) were purchased from Sigma. NAD (Cat. No. 481911) was obtained from CalBiochem (La Jolla, CA, USA). All other reagents for buffer solutions were obtained from Sigma. All reagents were used without further purification.

In order to investigate the effect of those reagents on the PheDH reaction, all measurements were started by adding the PheDH solution to the cuvette containing all other necessary reagents (see Results). The conversion to NADH at 5 min was monitored by a spectrophotometer (Lambda 2, Perkin Elmer, Norwalk, CT, USA) at 340 nm (ε=6.22 x 10^3 M^{-1} cm^{-1}) (5) and at room temperature.

For investigation of the effect of the reactants and products of the PheDH reaction on the bioluminescence system, bioluminescencent assays were started by adding the NADH solution into the tube containing all necessary reagents (see Results). In the assay for study of the pH effect and the buffer concentration effect, and for calibration curves, all assays were started by adding the Phe solution to the tube containing all other necessary reagents (see Results). The total integrated light emission at 5 min was measured by a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA) at room temperature and reported in relative light units (RLU).

All solutions were in phosphate or tris buffer of different pH or concentration (see Results). All reagent stock solutions were made freshly before assay and kept at 4 °C until use. Dodecanal was dissolved in methanol and used within 5 h. FMN and NADH solutions were also protected from the light.

Results and Discussion

A homogeneous-type biosensor, wherein all enzyme reactions are in progress at the same time and in the same volume, has fewer assay preparation steps. However, each enzyme competes for the substrates and products; the overall optimal reaction rate depends on the specific conditions.

The effects of the substrates and the products of each reaction on the other reactions must be determined and an optimal set of conditions for all reactions deduced in order to design a high performance homogeneous-type biosensor (6). The precise values of the substrates and the products in this system are not critical to the overall outcome (Figs 1 and 2); OR influenced the PheDH reaction, decreasing the output by about 20% (Fig. 1), and Phe, NAD, and PheDH influenced the bioluminescence reactions, decreasing the output by about 20% (Fig. 2). This ensures that the substrates and the products of each reaction do not significantly affect the other reaction when all substrates, except analytical one, are present in excess.

The effect of pH on the response in the homogeneous-type assay comes from two effects (7). Firstly, enzymatic activity is a function of pH and each enzyme has an unique optimal pH. Secondly, pH may affect the dissociation equilibrium of the product. Optimal pH of the bioluminescence reactions is pH 7.0 (8). However, PheDH from *Sporosarcina ureae* shows a pH optimum at pH 10.5 (9). The optimal pH of the overall assay system was pH 8.0 (Fig. 3). This pH value is closer to that of the bioluminescence reactions, suggesting that the PheDH reaction is driven by the NADH consumption of the bioluminescence reaction. In addition, the bioluminescence reaction is probably more sensitive to pH than is PheDH.

An optimal molarity of the buffer in the assay is also important. This effect was studied in phosphate and tris buffer (pH 8.0) with concentrations ranging from 10 mmol/L to 400 mmol/L. The highest light emission was showed in 50 mmol/L phosphate buffer (Fig. 4).

Optimal buffer concentration for the bioluminescence system is 100 mmol/L phosphate buffer (8). However, many different buffers can be used in the PheDH reaction. The bioluminescence reactions are probably more sensitive to the buffer condition than the Phe reaction.

The enzyme amounts needed for optimal assay are important in designing an economical and sensitive sensor. In the bioluminescent assay, 0.1 nmol of OR and LF were suitable for an assay volume of 250 µL (data not shown). For our system, the optimal enzyme amount of PheDH was 0.4 U (Fig. 5). However, the enzyme amounts needed for

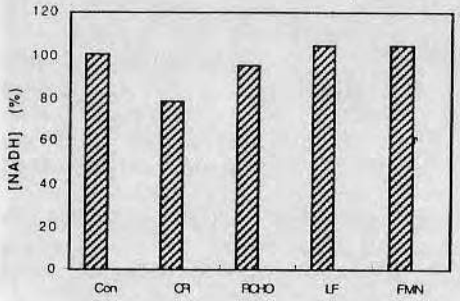

Fig. 1. The effects of the substrates and enzymes of the bioluminescence reactions on the PheDH reaction. FMN (1 µmol/L), dodecanal (0.0001% v/v), OR (1 nmol), or LF (1 nmol) were added, respectively, into the assay solution containing 1 mmol/L Phe, 1 mmol/L NAD, and 1 U PheDH in 100 mmol/L tris buffer (pH 9.0). The conversion to NADH at five minutes was monitored at 340 nm and at room temperature. Total assay volume was 1 mL. Thease data were average values (S.D.=5%, n=5). Con: control, OR: FMN:NAD(P)H oxidoreductase, RCHO: dodecanal, LF: bacterial luciferase, FMN: flavin mononucleotide.

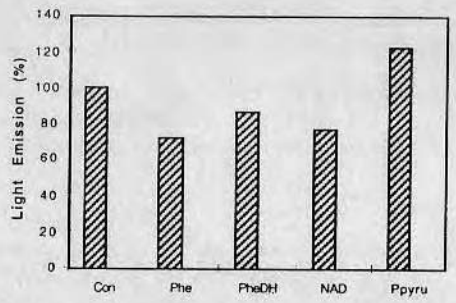

Fig. 2. The effects of the substrates, product, and enzyme of the PheDH reaction on the bioluminescence system. Phe (1 mmol/L), phenylpyruvic acid (1 mmol/L), NAD (10 mmol/L), or PheDH (1 U) were added, respectively, to the assay solution containing 10 µmol/L NADH, 1 µmol/L FMN, 0.0001% (v/v) dodecanal, 0.1 nmol OR, and 0.1 nmol LF in 100 mmol/L phosphate buffer (pH 7.0). The integrated light output at five minutes was measured by the luminometer at room temperature in relative light uints (RLU). Total assay volume was 250 µL. Thease data were average values (S.D.=5%, n=5). Con: control, Phe: phenylalanin, PheDH: phenylalanine dehydrogenase, NAD: nicotinamide adenine dinucleotide, Ppyru: phenylpyruvic acid.

optimal assay also depend on the assay volume (enzyme concentration) due to possible dimerization and aggregation effects.

Using the optimized conditions, the Phe concentration was assayed (Fig. 6). With 0.4 U PheDH, the linear range of the assay was from 0.1 µmol/L to 10 µmol/L Phe. The range covers 1 µmol/L to 1 mmol/L Phe using 0.04 U PheDH. The control level of light emission (without adding Phe) was close to the value for 0.1 µmol/L Phe (data not shown), due to the contamination level of Phe. This sensitivity is quite sufficient for Phe monitoring, because the reference range of clinical concern is generally 80-200 µmol/L (10). This information will be used in the development of a test-strip device for Phe monitoring in the home environment .

Acknowledgments

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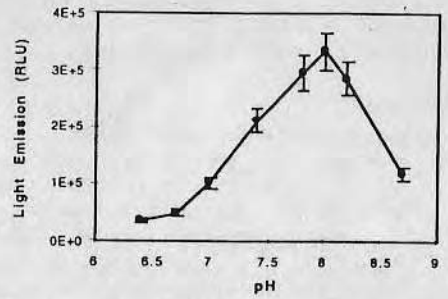

Fig. 3. The pH effect on the PheDH-bioluminescence system. Assay solutions contained 0.1 mmol/L Phe, 0.1 mmol/L NAD, 1 µmol/L FMN, 0.0001% (v/v) dodecanal, 0.1 nmol OR, and 0.1 nmol LF. Tris buffer (50 mmol/L) and 0.6 U PheDH were used. The integrated light output at five minutes was measured by the luminometer at room temperature in relative light uints (RLU) (S.D.=10%, n=4). Total assay volume was 250 µL.

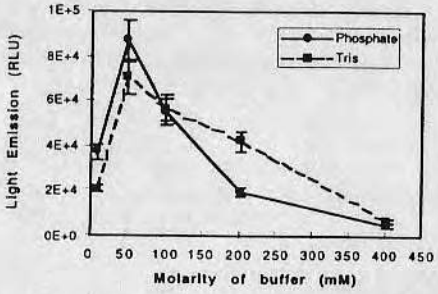

Fig. 4. The effect of buffer concentration on the PheDH-bioluminescence system. Assay solutions contained 0.1 mmol/L Phe, 0.1 mmol/L NAD, 1 µmol/L FMN, 0.0001% (v/v) dodecanal, 0.1 nmol OR, and 0.1 nmol LF. Tris and Phosphate buffers (pH 8.0) and 0.02 U PheDH were used. The integrated light output at five minutes was measured by the luminometer at room temperature in relative light uints (RLU) (S.D.=10%, n=4). Total assay volume was 250 µL.

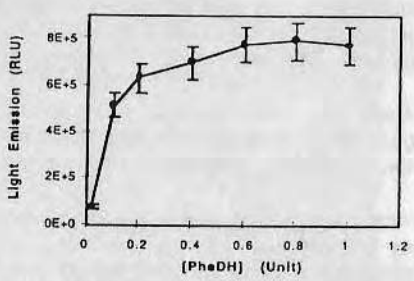

Fig. 5. The effect of PheDH activity on light emission in the PheDH-bioluminescence system. Assay solutions contained 0.1 mmol/L Phe, 0.1 mmol/L NAD, 1 µmol/L FMN, 0.0001% (v/v) dodecanal, 0.1 nmol OR, and 0.1 nmol LF. The buffer was 50 mmol/L phosphate (pH 8.0). The integrated light output at five minutes was measured by the luminometer at room temperature in relative light uints (RLU) (S.D.=10%, n=5). Total assay volume was 250 µL.

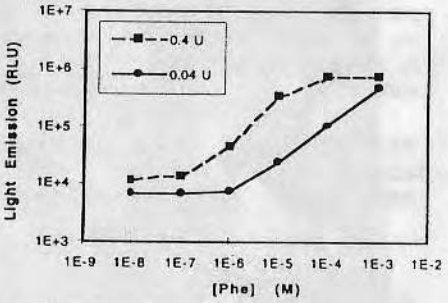

Fig. 6. The calibration curves for Phe using the PheDH-bioluminescence system. Assay solutions contained 0.1 mmol/L NAD, 1 µmol/L FMN, 0.0001% (v/v) dodecanal, 0.1 nmol OR, and 0.1 nmol LF. The buffer was 50 mM Phosphate (pH 8.0). The amount of PheDH was 0.4 U or 0.04 U. The integrated light output at five minutes was measured by the luminometer at room temperature in relative light uints (RLU) (S.D.=5%, n=5). Total assay volume was 250 µL. Both axes are log scale.

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Specific Immobilization of *in Vivo* Biotinylated Bacterial Luciferase and FMN:NAD(P)H Oxidoreductase

Dong J. Min,* Joseph D. Andrade,† and Russell J. Stewart*,†,[1]

*Department of Materials Science and Engineering and †Department of Bioengineering, 50 S. Campus Central Drive, Room 2480, University of Utah, Salt Lake City, Utah 84112-9202

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Bacterial bioluminescence, catalyzed by FMN: NAD(P)H oxidoreductase and luciferase, has been used as an analytical tool for quantitating the substrates of NAD(P)H-dependent enzymes. The development of inexpensive and sensitive biosensors based on bacterial bioluminescence would benefit from a method to immobilize the oxidoreductase and luciferase with high specific activity. Toward this end, oxidoreductase and luciferase were fused with a segment of biotin carboxy carrier protein and produced in *Escherichia coli*. The *in vivo* biotinylated luciferase and oxidoreductase were immobilized on avidin-conjugated agarose beads with little loss of activity. Coimmobilized enzymes had eight times higher bioluminescence activity than the free enzymes at low enzyme concentration and high NADH concentration. In addition, the immobilized enzymes were more stable than the free enzymes. This immobilization method is also useful to control enzyme orientation, which could increase the efficiency of sequentially operating enzymes like the oxidoreductase–luciferase system.

Bacterial luciferase (luciferase), a heterodimeric enzyme composed of α (40 kDa) and β (37 kDa) subunits, catalyzes the reaction between reduced flavin mononucleotide ($FMNH_2$), a long chain aldehyde, and molecular oxygen to yield a long-chain carboxylic acid, flavin mononucleotide (FMN), and blue–green light (1). In bioluminescent bacteria, $FMNH_2$ is generated by FMN:NAD(P)H oxidoreductase (oxidoreductase, 24.5 kDa), which catalyzes the reduction of FMN at the expense of reduced pyridine nucleotides (NAD(P)H) (2). $FMNH_2$ is also generated by NADH- and NADPH-specific oxidoreductases (30). In a coupled reaction of the oxidoreductase and luciferase, production of light by the luciferase is directly proportional to the NAD(P)H concentration at limiting concentrations of NAD(P)H (10). When the oxidoreductase–luciferase system is coupled to NAD(P)H-dependent enzymatic reactions, light production can be proportional to the concentration of substrate of the enzyme reaction, and thereby used as a sensitive method to quantify medically important metabolites (3). Coupled enzyme systems have been developed to measure glucose, lactate, malate, alanine, and phenylalanine (4, 5, 11). In practice, detection limit and linear range in the assays depended on assay conditions including enzyme and reagent purity and reaction efficiency (3).

To develop inexpensive and sensitive biosensors based on enzyme reactions coupled to bacterial bioluminescence, it is advantageous to immobilize the luciferase and oxidoreductase. Enzyme immobilization in biosensors generally offers the advantages of repeated use, increased stability, easier handling, and decreased cost (3, 9). In addition, coimmobilization of sequentially operating enzymes, including the oxidoreductase–luciferase system, has been shown to improve reaction efficiency leading to higher specific activities (3, 8–11, 15, 24–26, 28). For example, Wienhausen and DeLuca (11) showed that the coimmobilized enzymes produced 10–20 times more light than the individually immobilized enzymes. In these previous reports, luciferase and/or oxidoreductase was chemically conjugated on the solid materials, including glass, nylon, and Sepharose (3, 9, 10, 15). These traditional immobilization methods resulted in substantial loss of activity as a result of the immobilization procedure, resulting in inconsistent enzyme activity (3, 9–11). For example, Nabi and Worsfold (10) showed the activity recovery of oxidoreductase and luciferase chemically coupled to Sepharose beads was 33 and 14%, respectively. Wien-

[1] To whom correspondence should be addressed. Fax: (801) 581-8966. E-mail: rstewart@ee.utah.edu.

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hausen and DeLuca (11) also immobilized both enzymes chemically on Sepharose beads and found 40–90% activity recovery for oxidoreductase and 8–20% for luciferase.

As an alternative approach to conventional immobilization methods, we created biotinylated oxidoreductase and luciferase by genetic fusion with the biotin carboxy carrier protein (BCCP).[2] BCCP is one of three functional subunits of the acetyl-CoA carboxylase of *Escherichia coli* (12, 27). Biotin is attached posttranslationally to a specific C-terminal lysine residue of BCCP by the enzyme, biotin ligase (12). The C-terminal 87 residues of BCCP have been shown to be sufficient for biotinylation (12). Expression of the fusion protein in *E. coli* results in efficient biotinylation of the BCCP segment of the fusion protein. This approach has been previously applied to firefly luciferase (13).

Here we report that both luciferase and oxidoreductase fused with the BCCP segment were efficiently biotinylated *in vivo*. The *in vivo* biotinylated enzymes could be conveniently immobilized on avidin-conjugated beads without loss of activity. Furthermore, the coimmobilized system had a significantly higher specific activity than free enzymes.

MATERIALS AND METHODS

Construction of luxAB gene plasmids. Plasmid pJHD 500 containing *lux*AB gene from *Vibrio harveyi* was a gift from Dr. T. O. Baldwin (Texas A&M University). Plasmid pRSET-BCCP-*luc* had been previously constructed in our lab (13). To fuse the luciferase with a six-histidine tag (H-tag) (7), the *lux*AB gene was PCR-amplified from the pJHD 500 using the coding primer AAGGGATCCTTATGAAATTTGGAAACTTC and the noncoding primer GCCCTTTCGTCTTCAAGCTTTCTGTT, which have *Bam*HI and *Hin*dIII sites, respectively, for subcloning. The PCR product was digested with *Bam*HI and *Hin*dIII, gel-purified, and ligated to the expression vector pET-28C (Novagen, Madison, WI), which had been digested with *Bam*HI and *Hin*dIII. An H-tag was introduced onto the end of N-terminal side of α subunit of luciferase as a result of subcloning into pET-28C.

To construct the luciferase fusion protein with H-tag and BCCP segment, *lux*AB gene was obtained from pJHD 500 by PCR using the coding primer AATCTAGAAATGTTATGAAATTTGGAAACT (*Xba*I) and the noncoding primer GCCCTTTCGTCTTCAAGCTTTCTGTT (*Hin*dIII). The BCCP gene was obtained by PCR from pRSET-BCCP-*luc* using the coding primer CGCGGATCCGCATGGAAGCGCCAGCAGCAGCG (*Bam*HI) and the noncoding primer GCTCTAGATTCGATAACAACAAGCGGTTCGTC (*Xba*I). The PCR fragments digested with the appropriate enzymes were ligated simultaneously into pET-28C digested with *Bam*HI and *Hin*dIII. An H-tag and the BCCP segment were introduced onto the end of N-terminal side of α subunit of luciferase as a result of the subcloning.

Construction of oxidoreductase plasmids. To make H-tagged oxidoreductase, the *oxidoreductase* gene was PCR-amplified with the coding primer CGGGATCCCGCATCCAATTATTCATGAT (*Bam*HI) and the noncoding primer CGAATTCTGGCTTTTCTTCATCTCAAAC (*Eco*RI) from the genomic DNA of *Vibrio fischeri*. The PCR product was subcloned into the expression vector pRSET-C (Invitrogen, San Diego, CA) digested with *Bam*HI and *Eco*RI, which encodes an H-tag upstream of the multiple cloning sites.

To make the oxidoreductase fusion protein with H-tag and BCCP segment, the *oxidoreductase* gene was PCR-amplified with the coding primer GGTCTAGAATTATGACGCATCCAATTAT (*Xba*I) and the noncoding primer TTTCTTCAGCTGAAACATTAAGAATCTA (*Pvu*II) from the plasmid pRSET-oxidoreductase described above. The same PCR fragment of *BCCP* gene as above was used. The PCR products digested were ligated simultaneously to pRSET-C digested with *Bam*HI and *Pvu*II. An H-tag and the BCCP segment were introduced onto the N-terminal side of oxidoreductase as a result.

Enzyme expression. H-tagged luciferases, with and without the BCCP segment, were expressed in *E. coli* strain Nova Blue (DE3) (Novagen, Madison, WI). A single colony was inoculated into 50 ml Terrific Broth (TB) medium containing kanamycin and grown at 37°C overnight. Half of the 50-ml culture was transferred to 500 ml TB medium with kanamycin and further incubated at 37°C for 6 h (OD_{600} ≈5.0), and then induced at 30°C for at least 15 h with isopropyl β-D-thiogalactopyranoside (IPTG).

H-tagged oxidoreductases, with and without the BCCP segment, were expressed in *E. coli* strain BL21 (DE3). A single colony was inoculated into 50 ml Luria–Bertani (LB) medium containing ampicilin and incubated at 37°C overnight. Half of the 50-ml culture was transferred to 500 ml LB medium containing ampicilin and further cultured at 37°C for 3 h (OD_{600} ≈0.8), and then induced at 30°C for 4 h with IPTG.

Biotin (5 mg/500 ml medium) was added to 500 ml medium for the enzymes fused with the BCCP segment. The cells were pelleted at 6000 rpm, 4°C (JA-10 rotor, Beckman, Palo Alto, CA) for 20 min and stored at −70°C.

Enzyme purification. The frozen cell pellets were resuspended in lysis buffer (0.1 M phosphate, 0.5 M

[2] Abbreviations used: BCCP, biotin carboxy carrier protein; TB, Terrific Broth; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani; BSA, bovine serum albumin; RLU, relative light units; DTT, dithiothreitol.

NaCl, 10% (v/v) glycerol, 60 mM imidazole, 0.1% (v/v) Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 10 mM mercaptoethanol, pH 7.0). The resuspended cells were sonicated four times for 15 s on ice (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) and then centrifuged at 16,000 rpm, 4°C (JA-17 rotor, Beckman) for 30 min. The supernatant was applied to a Ni-NTA agarose column (Qiagen, Valencia, CA) preequilibrated with the wash buffer (0.1 M phosphate, 0.5 M NaCl, 10% (v/v) glycerol, and 60 mM imidazole, pH 7.0). The Ni-NTA column was washed four times with five packed bead volumes of the wash buffer and then eluted with the elution buffer (0.1 M phosphate, 0.5 M NaCl, 10% (v/v) glycerol, and 0.2 M imidazole, pH 7.0). Each fraction was monitored for protein on 12% SDS–PAGE. The positive fractions were pooled and buffer exchanged into 0.1 M phosphate (pH 7.0) using a prepacked Sephadex G-25 column (PD-10 column, Pharmacia Biotech, Piscataway, NJ). The desalted luciferases and oxidoreductases were concentrated with Centricon-30 and -3 (Amicon, Beverly, MA), respectively. The aliquots of the concentrated enzymes were frozen in liquid nitrogen and stored at −70°C until use. The concentration of enzymes was determined by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Assay for measuring the specific activity of enzymes. Specific activities of luciferases were determined by bioluminescence assay. The assay was started by adding of NADH (0.1 mM) to 1 μM FMN, 0.0001% (v/v) dodecanal, 24 μg oxidoreductase, and 2 ng luciferase in 0.1 M phosphate (pH 7.0) in a total assay volume of 250 μl. Light intensity was measured by a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA) at room temperature. Activity was reported in relative light units (RLU). The concentrations given are the final solution concentration.

Specific activities of oxidoreductases were measured by monitoring the NADH concentration spectrophotometrically (Lambda 2, Perkin Elmer, Norwalk, CT) at 340 nm ($\epsilon = 6.3 \times 10^3$ M^{-1} cm^{-1} (2)) at room temperature. The reaction was initiated by adding 1 μg oxidoreductase into a cuvette containing 0.1 mM NADH and 0.1 mM FMN in 0.1 M phosphate (pH 7.0). Total reaction volume was 1 ml.

Enzyme immobilization. Biotinylated luciferase and oxidoreductase were immobilized on the avidin-conjugated agarose beads (ImmunoPure Immobilized Avidin, Pierce, Rockford, IL) packed in a column. The beads were equilibrated twice with 10 packed bead volumes of 0.1 M phosphate (pH 7.0). For the immobilization of a single protein, excess biotinylated protein was applied into each column. For the immobilization of both proteins simultaneously (coimmobilization), amounts of biotinylated luciferase and oxidoreductase equal to the measured binding capacity of avidin-conjugated bead were mixed together and applied to the column. The column was then washed five times with 10 packed bead volumes of 0.1 M phosphate (pH 7.0). The bound enzymes were stored at 4°C until use.

Activities of free, individually immobilized and coimmobilized enzymes were determined by the bioluminescence assay with 10 pmol luciferase and 10 pmol oxidoreductase for each NADH concentrations from 1 nM to 0.5 mM, as above. For enzyme ratio effect in free and coimmobilized enzymes at 0.1 mM NADH, amounts of oxidoreductase were increased from 10 to 500 pmol at a fixed 10 pmol luciferase. The amount of coimmobilized enzymes was adjusted by the bead amount. The peak intensity was used for NADH calibration curves and for the enzyme ratio effect.

Stability of free and immobilized enzymes. Free and immobilized enzymes were stored at 4°C with and without dithiothreitol (DTT, 1 mM). At a given time, enzyme activity was measured by the bioluminescence assay, as above. For measuring the stability of luciferases, 10 pmol stored luciferase and 100 pmol fresh free oxidoreductase were used. For the stability of oxidoreductases, 10 pmol stored oxidoreductase and 100 pmol fresh free luciferase were used.

RESULTS

Purification and specific activity of luciferases and oxidoreductases. H-tagged luciferase, H-tagged oxidoreductase, H-tagged BCCP–luciferase, and H-tagged BCCP–oxidoreductase were expressed in *E. coli* and purified on a Ni-NTA column. Purity of the H-tagged luciferases and oxidoreductases was over 95%, as judged by SDS–PAGE (Fig. 1). Yield of luciferases and oxidoreductases was about 1.5 and 5.0 mg/l liter culture, respectively. The specific bioluminescence activity of the recombinant luciferases was comparable to commercially available luciferase (Boehringer) (Table 1), suggesting that the activity of luciferase was not inhibited by the H-tag or the BCCP segment. Likewise, the specific activity of recombinant oxidoreductases was comparable to commercial oxidoreductase (Boehringer) (Table 1) and to other reported oxidoreductase specific activities (2, 31), suggesting that the activity of oxidoreductase was not inhibited by the H-tag or the BCCP segment. However, our specific activity was lower than that reported by Inouye (29). This discrepancy may be due to different enzyme purity and assay method.

The extent of biotinylation of BCCP–luciferase and BCCP–oxidoreductase. The degree of biotinylation on BCCP–luciferase and BCCP–oxidoreductase was determined using an avidin-conjugated agarose column. Diluted enzyme solution, luciferase or oxidoreductase

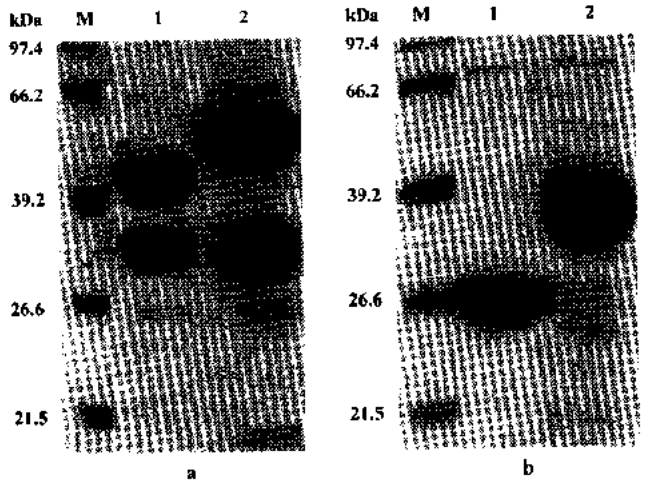

FIG. 1. SDS–PAGE (12%) of purified luciferases and oxidoreductases using the Ni-NTA column. The gels were stained with Coomassie blue. (a) Lane 1, luciferase; lane 2, BCCP–luciferase. (b) Lane 1, oxidoreductase; lane 2, BCCP–oxidoreductase.

(20 μg enzyme; much less than the bead's binding capacity as described below), was applied to the column (100 μl beads). The flowthrough was collected and reapplied to the column six times. The amount of unbound enzymes in the column flowthrough was determined by a Bio-Rad protein assay kit. The fraction of biotinylated luciferase and oxidoreductase was calculated, assuming all biotinylated enzymes were bound, by substracting the flowthrough from the initial amount of enzyme applied to the column (16). Nonspecific binding was found to be negligible in control experiments. The extent of biotinylation of BCCP fusion proteins expressed in *E. coli* can be different from culture to culture (20). To increase the extent of biotinylation of the fusion protein, biotin was added to the culture medium (20, 27). With biotin in the culture medium, luciferase and oxidoreductase were about 90 ± 5 and 75 ± 5% biotinylated (n = 3), respectively. Without biotin, the extent of biotinylation of luciferase and oxidoreductase was about 50 ± 10 and 40 ± 10% (n = 4), respectively.

The binding capacity of avidin-conjugated beads. The binding capacity of luciferase and oxidoreductase on the beads was determined by adding increasing amounts of enzyme to a fixed volume of beads to determine the bead saturation level. Solutions of each enzyme (22 to 534 μg luciferase and 50 to 593 μg oxidoreductase) were applied to the column (50 μl beads). The enzyme concentration in the flowthrough was determined. The binding capacity of the beads for luciferase and oxidoreductase was about 1.98 and 2.10 μg/μl, respectively (Fig. 2).

Immobilization of biotinylated luciferase and oxidoreductase. Biotinylated luciferase and oxidoreductase were immobilized together or individually on avidin-conjugated agarose beads. Since the biotin–avidin interaction is strong ($K_a = 10^{15}$ M^{-1}) (14), the immobilization of luciferase and oxidoreductase is quite stable. The specific activity of immobilized luciferase was measured by the same method as free enzyme, using a large molar excess of free oxidoreductase, and was 10 RLU/μg · s, about 80 ± 5% (n = 4) that of free BCCP–luciferase. The specific activity of immobilized oxidoreductase was measured spectrophotometrically, using 2 μg immobilized oxidoreductase, and was 4.7 μmol/mg · min, about 90 ± 5% (n = 4) that of free BCCP–oxidoreductase.

The bioluminescence activity of the coupled oxidoreductase–luciferase reaction was compared for free, coimmobilized, and individually immobilized enzymes, using 0.1 mM NADH, 10 pmol luciferase, and 10 pmol oxidoreductase (Fig. 3). The peak intensity of coimmobilized enzymes was about 8 times higher than that of free enzymes. The peak intensity of individually immobilized enzymes was about 20 times lower than that of free enzymes. These results were consistent with the results of DeLuca's group, who suggested that the microenvironments in the coimmobilized enzymes may protect $FMNH_2$ from autooxidation, resulting in more efficient $FMNH_2$ utilization by the luciferase (11, 17, 21). The time at the peak intensity was 10 s for free enzymes, 16 s for coimmobilized enzymes, and 60 s for singly immobilized enzymes. The reaction rate of immobilized enzymes might be slowed by the rate of mass transfer of the substrates from the bulk solution to the surface of the bead (9, 18, 23).

To study dependence of the microenvironmental effect on NADH concentration as well as to determine the effect of immobilization on the sensitivity and lin-

TABLE 1

Specific Activities of Luciferases and Oxidoreductases

	Specific activity (RLU/μg · s)		Specific activity (μmol/mg · min)
Boehringer luciferase	15.0	Boehringer oxidoreductase	3.5
Luciferase	10.0	Oxidoreductase	4.1
BCCP–luciferase	12.5	BCCP–oxidoreductase	5.3

Note. These data were average values (SD = 10%, n = 5). RLU, relative light units.

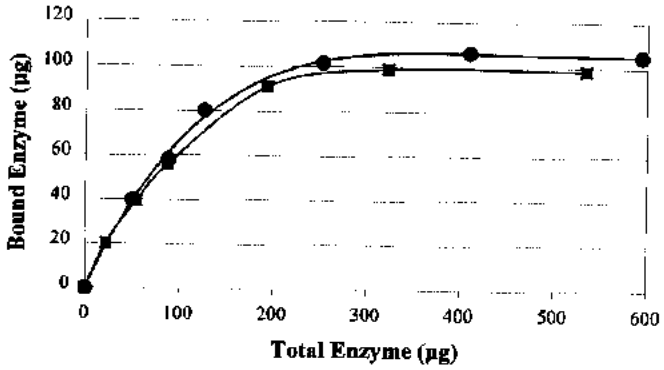

FIG. 2. The binding capacity of avidin-conjugated beads for BCCP–luciferase and BCCP–oxidoreductase. The percentages of biotinylation of luciferase and oxidoreductase used were about 92 and 79%, respectively, and the bead volume was 50 μl. The binding capacity of the beads for luciferase and oxidoreductase was about 1.98 and 2.10 μg/μl, respectively. The amount of enzymes was determined by a Bio-Rad protein assay kit using BSA as a standard. These data were average values (SD = 5%, n = 4). ■, BCCP–luciferase; ●, BCCP–oxidoreductase.

earity, the same experiments were performed with NADH concentrations from 1 nM to 0.5 mM (Fig. 4). The linear range was between 1 nM and 1 μM for free enzymes, 1 nM and 10 μM for coimmobilized enzymes, and 0.1 and 10 μM for individually immobilized enzymes. At concentrations below 1 μM NADH, the peak intensity of coimmobilized enzymes was slightly higher than that of free enzymes. NADH diffusion through the agarose beads in the coimmobilization may be limited to produce light at the low NADH concentrations.

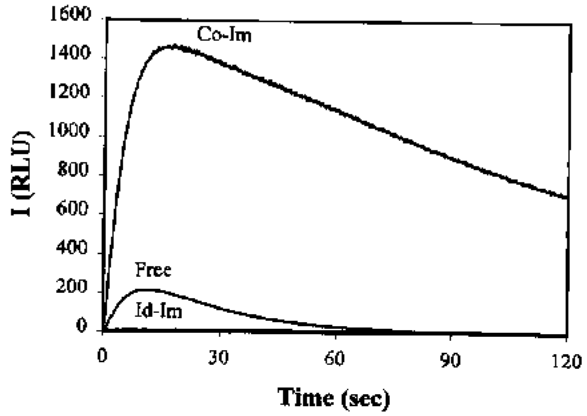

FIG. 3. Bioluminescence assays for free, coimmobilized, and individually immobilized, luciferase and oxidoreductase. Assay was started by adding NADH (0.1 mM) to 1 μM FMN, 0.0001% (v/v) dodecanal, 10 pmol oxidoreductase, and 10 pmol luciferase in 0.1 M phosphate (pH 7.0) in a total assay volume of 250 μl. The light intensity was measured by the luminometer at room temperature and was reported in relative light units (RLU). Co-Im, coimmobilized enzymes; Free, free enzymes; Id-Im, individually immobilized enzymes.

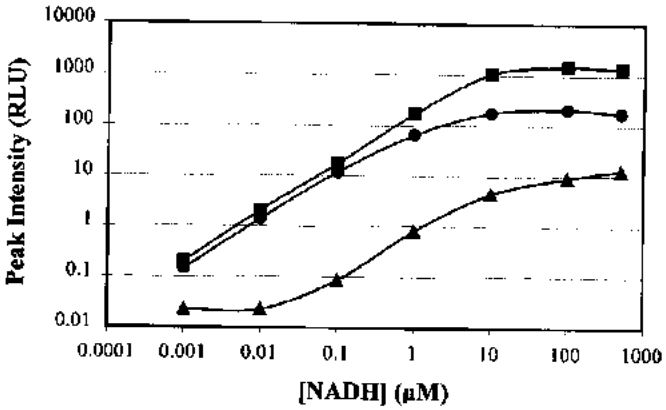

FIG. 4. Calibration curves of bioluminescence intensity vs NADH concentration for free, coimmobilized, and individually immobilized luciferase and oxidoreductase. Assay was started by adding different NADH (1 nM to 0.5 mM) to 1 μM FMN, 0.0001% (v/v) dodecanal, 10 pmol oxidoreductase, and 10 pmol luciferase in 0.1 M phosphate (pH 7.0) in a total assay volume of 250 μl. The peak intensity was measured by the luminometer at room temperature and was reported in relative light units (RLU). These data were average values (SD = 5%, n = 4). Both axes are log scale. ■, coimmobilized enzymes; ●, free enzymes; ▲, individually immobilized enzymes.

In the oxidoreductase–luciferase system, the mean distance between luciferase and oxidoreductase is probably important to produce light efficiently due to the $FMNH_2$ autooxidation. The distance between both enzymes is shorter in the coimmobilization system than that in the free enzyme system at low enzyme concentrations. To test this hypothesis, the distance between both enzymes was decreased by increasing the oxidoreductase concentration at a fixed luciferase concentration, 10 pmol. The bioluminescence peak intensity with ratios of 1, 8, 13, 29, 39, and 50 mol/mol of free or coimmobilized oxidoreductase to luciferase was determined (Fig. 5). The peak intensity was almost the same for free or coimmobilized enzymes at ratios above 1 mol/mol. In addition, the peak intensity plateaued at ratios higher than 30 for both cases. These results suggest that at high ratios both free and coimmobilized enzymes are in similar environments and that the coimmobilization is a good method to decrease the mean distance at the dilute enzyme concentration. To produce the highest peak intensity for a given luciferase concentration, the oxidoreductase concentration needed is 30 times more than the luciferase concentration in the free enzymes and may depend on immobilization conditions in the coimmobilized enzymes.

Stability of free and immobilized luciferase and oxidoreductase. One of the potential advantages of immobilized enzymes is their increased stability (8). To compare the stability of free and immobilized BCCP–luciferase and BCCP–oxidoreductase, the enzymes were stored at 4°C with and without DTT, and their activities were measured periodically (Table 2). Immo-

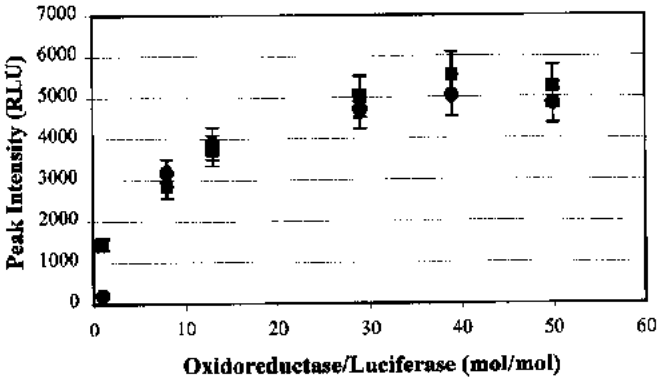

FIG. 5. The enzyme ratio effects for free and coimmobilized luciferase and oxidoreductase. The amount of oxidoreductase was increased from 10 to 500 pmol at a fixed 10 pmol luciferase. Assay was started by adding NADH (0.1 mM) to 1 μM FMN, 0.0001% (v/v) dodecanal, oxidoreductase (10 to 500 pmol), and 10 pmol luciferase in 0.1 M phosphate (pH 7.0) in a total assay volume of 250 μl. The peak intensity was measured by the luminometer at room temperature and was reported in relative light units (RLU). ■, coimmobilized enzymes; ●, free enzymes.

bilized enzymes were generally more stable than the free enzymes. For example, free luciferase and oxidoreductase without DTT lost half of their activity after 1 and 3 days, respectively, but immobilized luciferase and oxidoreductase without DTT was stable for 14 and over 21 days, respectively. Addition of DTT had a significant effect on the free enzymes but had little influence on the immobilized enzymes. Oxidoreductase is more stable than luciferase.

DISCUSSION

Specific, sensitive, and rapid bioluminescent assays for NAD(P)H have been used as an analytical tool for measuring several medically important analytes (3, 11). As part of the development of these diagnostic assays, luciferase and oxidoreductase have been expressed in *E. coli* and immobilized on solid materials in several labs (2, 3, 6, 8, 10, 15). The immobilization of oxidoreductase and luciferase by the methods described in these reports generally resulted in low binding efficiency, with significant loss of enzyme activity (3, 10, 11). An alternative approach is the avidin–biotin system, which has been widely applied to enzyme immobilization due to its specific and strong bond (14).

Conventional enzyme biotinylation usually involves biotin introduced chemically through a functional group of the enzyme. Chemical biotinylation can result in multiple biotinylation, random biotinylation, and enzyme inactivation (16). Li and Cronan (27) introduced *in vivo* enzyme biotinylation of proteins fused with BCCP. There are several advantages of *in vivo* biotinylation of proteins: the BCCP fusion proteins are biotinylated in the cell as posttranslation modifications, eliminating the need for chemical modification; the biotinylation occurs on a specific residue of the BCCP segment; the enzymes have a consistent orientation on the solid surfaces; the orientation can be controlled to some extent by selecting the fusion site with BCCP segment; and the BCCP segment between the avidin binding site and the enzyme molecule may act like a spacer, preventing enzyme denaturation resulting from interactions with the surface.

Bacterial luciferase and oxidoreductase fused with the *in vivo* biotinylated BCCP segment could be conveniently immobilized on avidin-conjugated agarose beads. This approach was highly effective at preserving the activity of both enzymes, which in the past have been shown to be sensitive to surface immobilization.

TABLE 2

Stability of Free and Immobilized Enzymes

	Activity retained (%)							
	BCCP–luciferase				BCCP–oxidoreductase			
	Free		Immobilized		Free		Immobilized	
Time (day)	With DTT	Without DTT	With DTT	Without DTT	With DTT	Without DTT	With DTT	Without DTT
0[a]	100	100	100	100	100	100	100	100
1	15.1	2.7	94.8	94.1	82.0	68.2	99.9	99.9
3	6.2	1.7	90.7	92.0	81.0	43.6	99.6	99.7
7	5.0	0.2	84.4	88.4	79.5	43.2	99.6	99.7
14	5.0	0.1	74.9	83.6	73.4	42.7	99.6	98.6
21	—	—	6.0	8.0	73.0	38.2	89.3	85.0

Note. Activity was determined by the peak intensity at room temperature. These data were average values (SD = 5%, $n = 4$).
[a] Activities of fresh enzymes were used as initial values. The storage temperature was 4°C.

Furthermore, coimmobilization resulted in an eightfold increase in specific activity relative to free enzymes. In NADH assays with the coimmobilized enzymes, light emission was linear over an NADH concentration range from 1 nM to 0.5 mM. This suggests that sensitive biosensors with a wide useful detection range can be developed using the BCCP–luciferase and –oxidoreductase system.

For application in biosensors, coimmobilization of oxidoreductase and luciferase is also of interest because of the increased coupling efficiency between the enzymes (3, 8). The increased efficiency is due to a decrease in nonenzymatic oxidation of $FMNH_2$, which competes with production of light through enzymatic oxidation of $FMNH_2$. The probability of nonenzymatic oxidation of $FMNH_2$ decreases as the effective concentration of oxidoreductase and luciferase increases through coimmobilization, resulting in increased light intensity (assay sensitivity) at low enzyme concentrations compared to enzymes free in solution (Fig. 3) (9). A similar phenomenon may occur in nature; Tu and Hastings (22) suggested that *in vivo* luciferase and oxidoreductase exist as a complex. For both free and coimmobilized enzymes the coupling efficiency increased at higher ratios of oxidoreductase to luciferase, saturating at about 30:1 (Fig. 5).

In addition to higher specific activities at low enzyme concentration, the immobilized luciferase and oxidoreductase were also more stable than the free enzymes. The increased stability of immobilized enzymes may be a general phenomenon, observed with several enzymes and immobilization methods (9, 11, 15, 19). Combes *et al.* (19) suggested that increased organization of solvent molecules in the vicinity of a solution–solid interface may prevent unfolding of proteins. Enhancement of the stability of the luciferase and oxidoreductase by immobilization on avidin surfaces may be important for increasing biosensor stability and shelf life.

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