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Properties of Bacterial Luciferase/NADH : FMN Oxidoreductase and Firefly Luciferase Immobilized onto Sepharose

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Abstract

NADH : FMN oxidoreductase and bacterial luciferase have been efficiently co-immobilized onto Sepharose 4B. This luminescent immobilized enzyme system can be used to assay NADH. The assay is rapid and sensitive with a lower limit of detection of 0.2 pmol/assay tube. The intra-assay precision was 3.5% at $2 \times 10^{-5}M$ and 5.8% at $2 \times 10^{-6}M$ NADH. Light intensity was proportional to NADH concentration from 0.2 to 1000 pmol. Added serum and certain dehydrogenases were found to be inhibitory; however, inhibition could be eliminated by a combination of heat treatment and dilution.

Firefly luciferase has also been immobilized onto both Sepharose 4B and CL 6B. The detection limit for ATP using this immobilized enzyme was 0.2 pmol and the assay was linear from 0.2 to 2000 pmol. The intra-assay precision was 4.8% at $2 \times 10^{-4}M$ and 3.2% at $1 \times 10^{-5}M$ ATP.

The immobilized enzymes remained fully active when rapidly frozen in the presence of glycerol and DTT. Such preparations could be stored for at least two months with no loss of activity. A variety of different compounds were used to block any remaining reactive groups on the Sepharose following immobilization of the enzymes.

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Glycine, 2-aminoethanol, and ethylenediamine were examined. The preparations where ethylenediamine was used as a blocking agent exhibited better activity and stability than the others.

Index Entries: Immobilized NADH : FMN oxidoreductase, and bacterial luciferase; immobilized firefly luciferase; analytical interferences, effect of freezing and thawing on; blocking groups; Sepharose; bioluminescent NADH and ATP assays; stability of immobilized enzymes, bacterial luciferase, immobilized; luciferase, immobilized firefly; firefly luciferase, immobilized; oxidoreductase, immobilized.

Introduction

Luminescent marine bacteria contain an NADH : FMN oxidoreductase and luciferase that catalyze the following reactions:

$$H^+ + NADH + FMN \xrightarrow{\text{oxidoreductase}} NAD^+ + FMNH_2$$

$$FMNH_2 + \text{decanal} + O_2 \xrightarrow[\text{luciferase}]{\text{bacterial}} FMN + \text{decanoic acid} + H_2O + \text{light}$$

The oxidoreductase oxidizes NADH with the formation of stoichiometric amounts of $FMNH_2$. The bacterial luciferase catalyzes the reaction of $FMNH_2$ with decanal and oxygen to produce FMN, decanoic acid, and light. The amount of light produced is proportional to the NADH concentration when it is present in limiting quantities. This coupled system offers a convenient and sensitive method for measuring NADH or NADPH. The enzymes have been immobilized on glass beads (*1*) and with greater efficiency onto Sepharose 4B (*2*). Coimmobilization of several other NAD dependent dehydrogenases along with the oxidoreductase and bacterial luciferase has also been reported (*2*). These immobilized enzymes have been used to assay clinically important compounds such as glucose, androsterone, testosterone (*2*) and primary bile acids (*3*).

Luciferase from the firefly, which catalyzes the reaction of ATP with luciferin, has also been immobilized onto both cellophane and Sepharose (*4, 5*). The immobilized enzyme has been used for a bioluminescent assay of ATP, but this application has not been extensively studied.

$$ATP + \text{luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{firefly luciferase}} AMP + \text{oxyluciferin} + PP_i + \text{light}$$

The analytical usefulness of such bioluminescent assays is dependent upon the properties of the immobilized enzymes. This paper describes results of studies of the basic properties of the immobilized oxidoreductase, bacterial luciferase, and firefly luciferase. The stability, sensitivity, precision, and the effects of interfering substances and the microenvironment of the immobilized enzymes have been examined.

Materials and Methods

Glycine, FMN, decanal, ethylenediamine dihydrochloride, and dithiothreitol (DTT) were purchased from Sigma Company (St. Louis, Missouri, 63178). Cyanogen bromide and 2-aminoethanol were obtained from Eastman Chemical Company (Rochester, New York, 14650). Lactate dehydrogenase (pig heart, 3950 IU/L), TES, adenosine 5′-triphosphate, NAD, and NADH were supplied by Calbiochem Behring Corporation (La Jolla, California, 92037). Sepharose 4B and CL 6B were obtained from Pharmacia Fine Chemicals (Piscataway, New Jersey 08854). Glycerol was purchased from Mallinckrodt, Inc. (Paris, Kentucky 40361).

Luciferase was isolated from a frozen cell paste of *Beneckea harveyi* strain B-392 (6) and NADH : FMN oxidoreductase was purified according to the method of Jablonski and DeLuca (7). The G-100 purified enzymes were stored at −70°C in 0.1*M* phosphate buffer (pH 7.0) containing DTT (2 m*M*).

Firefly luciferase was purified according to the procedure of Green and McElroy (8). Luciferin was synthesized as described previously (9).

Immobilization Procedure

Bacterial luciferase/NADH : FMN oxidoreductase and firefly luciferase were immobilized onto cyanogen bromide activated Sepharose 4B or Sepharose CL 6B as described by Ford and DeLuca (2). A minor modification to this method was that the bicarbonate buffer was replaced throughout by sodium pyrophosphate (0.1*M*, pH 8.0). The immobilized enzymes were stored in phosphate buffer (0.1*M*, pH 7.0) containing BSA (2 mg/mL) azide (0.2 mg/mL) and 2 m*M* DTT.

Effects of Different Blocking Reagents on the Properties of Immobilized Bioluminescent Enzymes

Reactive groups remaining on cyanogen bromide activated Sepharose after exposure to the bioluminescent enzymes are usually blocked with an amine. Glycine has been used for this purpose, and it introduces negatively charged groups onto the Sepharose. The effect on enzyme activity of blocking reagents that introduce positively charged, negatively charged, and neutral blocking groups onto the Sepharose was investigated using ethylenediamine dihydrochloride, glycine, and 2-aminoethanol, respectively. A batch of immobilized enzymes was prepared and 1 g portions were suspended in 10 mL of 2*M* solutions of the various blocking reagents in pyrophosphate buffer (0.1*M* pH 8.0). These were incubated at 4°C for 2 h and then extensively washed as described previously (2).

pH-Activity profiles of immobilized bacterial luciferase/oxidoreductase and firefly luciferase were determined using 0.1*M* phosphate buffer and 0.1*M* TES buffers, respectively, in the pH range 6.5–8.5.

Coupled NADH : FMN Oxidoreductase/Bacterial Luciferase Assay

A decanal–water emulsion was prepared by shaking 5 μL of decanal with 10 mL water. This was prepared daily and stored at 0–4°C. Decanal emulsion (100 μL),

200 μL aqueous FMN (73 μ*M*), 1 mL aqueous NAD (0.02*M*) and 10 mL phosphate buffer (0.1*M*, pH 7.0), were mixed together and stored at room temperature in the dark. This solution is stable for 3–4 h. A 50 μL aliquot of immobilized enzymes (17 mg Sepharose/mL) and 500 μL of the assay buffer were mixed together in a glass tube. NADH (50 μL) was added and the contents of the tube mixed twice by inversion. Peak ligh emission, which occurred *ca.* 1 min after the start of the reaction, was measured using an Amino Chem-Glow photometer (Aminco, Division of Travenol Laboratories, Inc., Silver Spring, Maryland, 20910). Assays were carried out at ambient temperatures (18–20°C). All assays were done in quadruplicate.

Bacterial Luciferase Assay

Decanal buffer was prepared from 100 μL decanal emulsion and 9.9 mL phosphate buffer (0.1*M*, pH 7.0). $FMNH_2$ was produced by photo-reducing 100 μL aliquots of a solution of FMN (0.15 m*M*) in deoxygenated phosphate buffer (0.1*M*, pH 7.0) containing EDTA (5 m*M*) (*10*). This solution was injected into a mixture containing 50 μL immobilized enzymes (17 mg/mL) and 500 μL decanal buffer and the peak light emission was recorded.

NADH : FMN Oxidoreductase Assay

Sepharose-bound oxidoreductase activity was measured spectrophotometrically, as described previously (*11*).

Firefly Luciferase Assay

The stock assay mixture comprised 8 mL glyclyglycine (0.25*M*, pH 7.8), 1.0 mL magnesium chloride (0.1*M*, pH 7.8) and 0.8 mL luciferin ($10^{-3}M$). A 10 μL aliquot of immobilized enzymes (10 mg/mL) or soluble luciferase was added to 400 μL assay buffer and 100 μL of ATP solution injected. Peak light intensity was recorded.

Matrix Effects. BSA; Solutions of NADH ($2 \times 10^{-4}M$) in PBS (0.015*M* phosphate buffer, pH 7.4, sodium chloride 8 mg/mL) and ATP ($2 \times 10^{-5}M$) containing different concentrations of BSA (1–100 mg/mL) were assayed in duplicate using 50 μL immobilized enzymes (17 mg Sepharose/mL). Solutions of NADH (2×10^{-4}) in PBS and ATP ($2 \times 10^{-5}M$) in TES buffer (0.01*M*, pH 7.6) were used as controls.

Human Serum. Solutions of NADH ($2 \times 10^{-4}M$) were prepared in a fresh human serum (total protein concentration *ca.* 70 mg/mL) and in 2-, 10-, and 100-fold dilutions of the serum in PBS. These solutions were assayed in duplicate for NADH concentration. A similar experiment was performed using the serum after it had been heated at 60–65°C for 0.5h.

Lactate Dehydrogenase. A solution of NADH (500 μL, $2 \times 10^{-4}M$) in PBS and in PBS containing BSA (80 mg/mL) were assayed in duplicate before and after addition of lactate dehydrogenase (50 μL, 3950 IU/L).

Stability of Immobilized Enzymes. Bacterial Luciferase/Oxidoreductase. 50 μL aliquots of immobilized enzymes were used to assay a solution of NADH (50 μL, 2×10^{-5} *M*) after the immobilized enzymes had been stored for varying periods of time in the presence of either 0.1 or 2 m*M* DTT under the following conditions: (i) 0–4°C, (ii) ambient temperature, 18–20°C, (iii) 56°C.

The effect of freezing (slowly at −20°C and quickly at −196°C) and thawing on the activity of immobilized enzymes in different storage buffers containing either 0.1 or 2 m*M* DTT with and without added glycerol (150 mg/mL) was also studied.

NADH : FMN oxidoreductase is the least stable of the two enzymes coimmobilized onto Sepharose. Thus for purposes of comparison the stability at ambient temperature of the soluble oxidoreductase (0.6 mg/mL, 2.2U) in the presence of luciferase (5 mg/mL) and BSA (4 mg/mL) was studied. (Luciferase and albumin were included in order to mimic the coimmobilized enzyme–BSA mixture.) A 10 μL portion of the enzyme solution was assayed for oxidoreductase activity as described previously (*7*).

Firefly Luciferase. The stability of immobilized firefly luciferase after freezing (−20 and −196°C) and thawing was studied using a storage buffer containing glycerol (150 mg/mL) and DTT (0.1 m*M*).

Results

Immobilized Bacterial Luciferase/Oxidoreductase

The amount of active enzyme obtained after immobilization onto Sepharose was determined for seven different immobilized preparations. This is expressed as a percentage of the total initial activity added to the Sepharose and ranged from 26 to 51% for the oxidoreductase and 6 to 19% for luciferase. The activities recovered by immobilization to cyanogen bromide activated Sepharose were far superior to other solid supports examined. These included different Enzacryls, CH-Sepharose 4B, Matrix 102, all with a variety of different reactive groups such as aldehyde, diazonium salt, and *N*-hydroxy succinimide ester. All of these resulted in preparations containing 0.1–0.01% of the active enzyme when compared to Sepharose 4B.

NADH Determination

Using standard assay conditions, a linear response of peak light intensity vs NADH concentration was obtained between 2 and 1000 pmol of NADH. Above 1000 pmol the assay was no longer linear. The lower detection limit of NADH was dependent upon the batch of immobilized enzyme used. This happens because each preparation has a different amount of background light associated with it. The lower limits ranged from 0.2 to 3.5 pmol/assay tube.

Figure 1 illustrates the time course of light emission after the addition of 50 pmol of NADH. The slight increase of light intensity observed after remixing occurs because the Sepharose slowly settles out and is resuspended upon mixing.

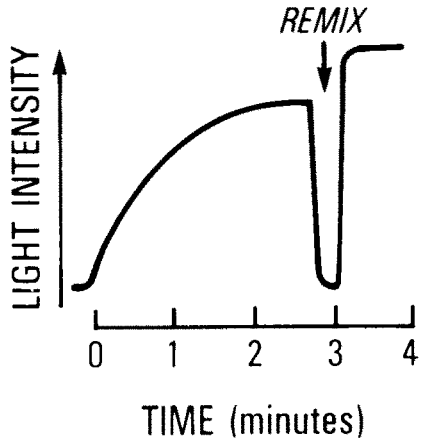

Fig. 1. Typical time course of light emission from reaction of $2 \times 10^{-6}M$ NADH (50 μL) with co-immobilized bacterial luciferase/oxidoreductase (50 μL, 17 mg Sepharose/mL). Effect on light emission of remixing the assay mixture is also shown. Subsequent remixing brings the emitted light back to the same relative intensity.

The precision of the assay based on nine replicates during a 1-day period was 3.4% at $2 \times 10^{-5}M$ NADH and 5.8% at $2 \times 10^{-6}M$ NADH.

Figure 2 shows the data obtained when increasing amounts of immobilized enzyme were added to a constant amount of NADH. Using either 1 or 20 pmol of NADH, a linear increase in light vs immobilized enzyme concentration was observed from 5 to 50 μL.

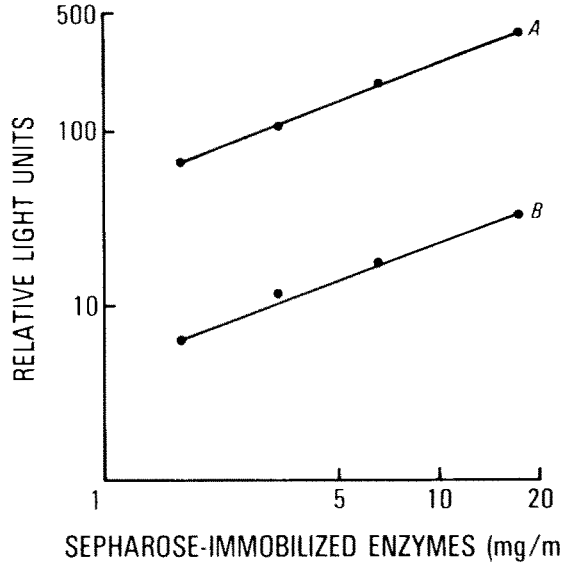

Fig. 2. Influence of immobilized enzyme concentration on peak light emission in an NADH assay. A, 10 pmol NADH; B, 1 pmol NADH. Concentration of Sepharose-immobilized enzymes shown is final concentration in assay tube.

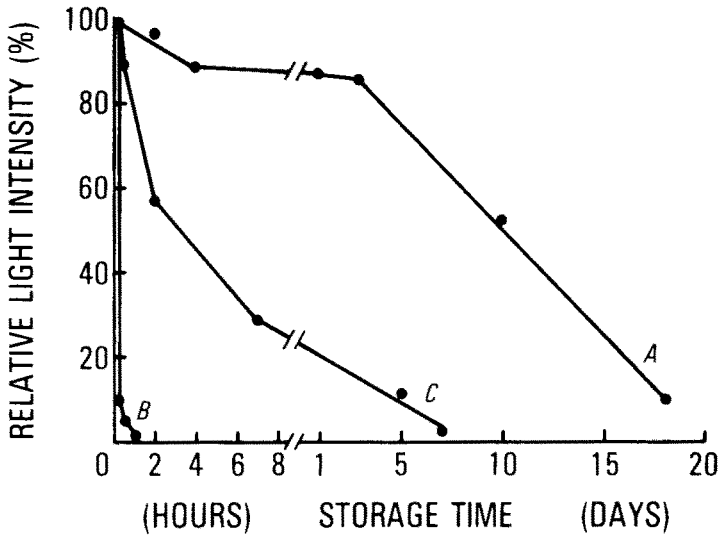

Fig. 3. Effect of storage on the coupled enzyme activity of glycine-blocked coimmobilized bacterial luciferase/oxidoreductase at ambient temperature (A), and at 56°C (B). Stability of soluble NADH : FMN oxidoreductase (C) is also shown for purposes of comparison.

Stability Studies

The immobilized enzymes exhibited good stability when stored at 0–4°C in a storage buffer containing 0.1 m*M* DTT, retaining 80% of the initial activity or better for 3 days. The concentration of DTT in the storage buffer is important for maintaining activity, and in the presence of fresh DTT (>0.2 m*M*) the immobilized enzymes were fully active after several months. At 56°C, 90% of the coupled enzyme activity was lost within 15 min. The loss of activity of a comparable concentration of the soluble enzyme was more rapid than the immobilized form at all temperatures, Fig. 3.

Repeated freezing of the immobilized enzymes at −20°C resulted in considerable loss of activity (60–75% loss in activity after three cycles or freezing and thawing). However, if they were frozen rapidly in liquid nitrogen at −196°C, there was an initial loss of activity, 10%, during the first freeze–thaw cycle and no further loss was observed. If 15% glycerol was added prior to freezing, there was no loss of activity and the immobilized enzymes could be stored frozen for at least 2 months with complete retention of activity.

Microenvironment Effects

The effect of the charge on the Sepharose was examined by quenching the unreacted sites with various compounds. The use of glycine results in a negative charge, 2-aminoethanol will be neutral, and ethylenediamine will confer a slightly positive charge at neutral pH. The initial immobilized enzyme activities obtained were the same with all three blocking reagents.

pH Activity profiles for the different blocked immobilized enzymes after storage at pH 7.0 are shown in Fig. 4. Similar profiles were observed with immobilized

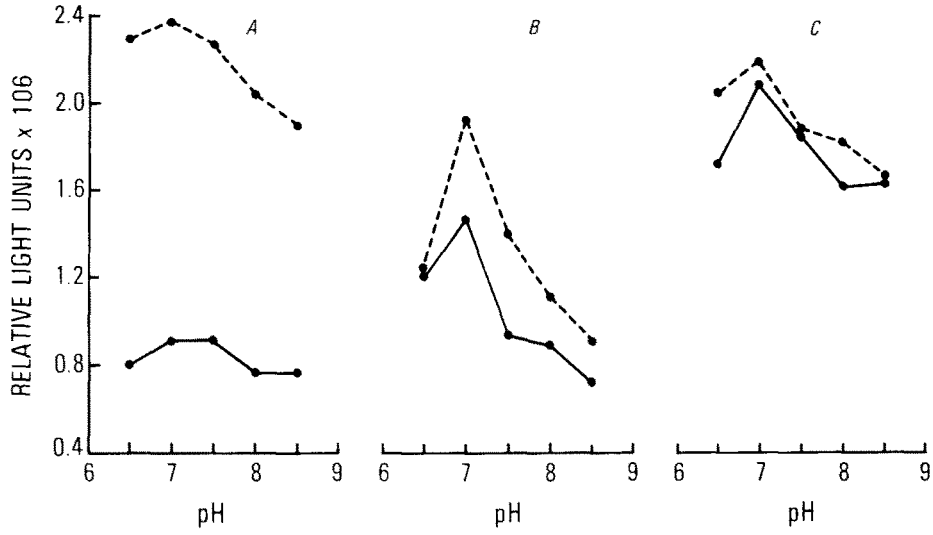

Fig. 4. pH-Activity profiles of coimmobilized bacterial luciferase/oxidoreductase blocked with glycine (A), 2-aminoethanol (B), and ethylenediamine (C). (___ in 0.1 m*M* DTT, _ _ _ _ in 2 m*M* DTT.)

enzymes that had been stored up to 40 days. The effect of high DTT concentrations (2 m*M*) was most pronounced for those blocked using glycine. At low DTT concentrations (0.1 m*M*) the activity of the glycine-blocked immobilized enzymes was 50–100% lower than comparable 2-aminoethanol or ethylenediamine-blocked preparations. Repeated slow freezing at −20°C and thawing decreased the activity, in particular for immobilized enzymes that had been blocked with 2-aminoethanol (90% loss in activity after four cycles of freezing and thawing). Ethylenediamine blocking groups conferred the most stability on the immobilized enzymes (55% loss in activity after four cycles of freezing and thawing). High concentrations of DTT did not protect the immobilized enzymes from the effects of repeated freezing and thawing.

Matrix Effects

BSA exhibited a concentration-dependent inhibition of light emission. At a BSA concentration of 80 mg/mL a 40% inhibition was observed. A similar trend was found with heat-treated human serum. Fresh human serum also inhibited light emission, but to a greater extent, e.g., serum inhibited light output by 73% compared with 40% for the same heat-treated serum. Lactate dehydrogenase also inhibited light output. The presence of lactate dehydrogenase (395 IU/L) in either an aqueous solution of NADH or a solution of NADH containing BSA (80 mg/mL) caused 27 and 30% inhibitions of peak light emission, respectively. This inhibition could be prevented by heating the sample at 60–65°C for 30 min.

Immobilized Firefly Luciferase. The activities recovered on the different preparations ranged from 44 to 84%. Highest recoveries were obtained when ethylenediamine was used as the blocking reagent. Inclusion of BSA in the coupling mixture gave only a slight improvement in recovered activity, e.g.,

ethylenediamine-blocked immobilized firefly luciferase had a recovered activity of 84% (+BSA), 73% (−BSA).

ATP Determination. A linear response of peak light intensity vs ATP concentration was observed between 200 fmol and 2 nmol of ATP. The detection limit for ATP using immobilized firefly luciferase prepared with BSA and blocked with ethylenediamine was 200 fmol/assay tube.

Stability Studies. The stability of immobilized firefly luciferase was only studied under the conditions found to be most favorable for coimmobilized bacterial luciferase/oxidoreductase, i.e., storage buffers containing glycerol and DTT. No loss in immobilized firefly luciferase activity was observed after freezing (−196°C) and thawing.

Microenvironment Effects. The different blocking reagents did not affect the shape of the pH-activity profile or its optimum (pH = 7.5).

Matrix Effects. Above 10 mg/mL, BSA inhibited light emission. A 22 and 45% inhibition of peak light emission was observed from ATP solutions containing 50 and 100 mg/mL of BSA, respectively.

Discussion

The different immobilized enzymes showed good sensitivity and a wide range of linear response for NADH and ATP.

The precision of these manual assays was acceptable and could very likely be improved by automation of the various dispensing and mixing steps. Accurate dispensing of particulate suspensions of immobilized enzymes is difficult and an obvious source of error in any assay based on this reagent. An estimate of the imprecision arising from inaccurate dispensing of immobilized enzymes on the light intensity obtained with high and low concentrations of NADH can be obtained from the data presented in Fig. 2. In this study the effect of variation in concentration was minimized by diluting the immobilized enzymes, thus allowing a large volume (50 μL) to be dispensed with improved accuracy and precision.

Many analytical methods are subject to interferences (matrix effects) from substances present in the test sample (*12*) and methods using immobilized enzymes are no exception. High concentrations of protein (e.g., BSA) inhibited light output, possibly as a result of binding of NADH or ATP to the BSA, thus making it inaccessible to the immobilized enzymes. Components of serum also inhibited the detection of NADH, and this is most likely a result of the consumption of NADH by dehydrogenases. Results obtained with solutions of lactate dehydrogenase support this notion. The reverse situation, i.e., production of NADH from NAD by dehydrogenase enzymes in serum, has also been observed in an assay for bile acids using coimmobilized bioluminescent enzymes and 7-α-hydroxysteroid dehydrogenase (*3*). In both cases the enzyme interference could be abolished by a brief heat treatment and 50-fold dilution of serum specimens.

Any reagent intended for routine analytical use should be stable. The immobilized enzymes showed good stability at both 0–4°C and at ambient temperature and

in common with other enzymes (*13*) immobilized NADH : FMN oxidoreductase and immobilized firefly luciferase were more stable than their soluble counterparts. Inclusion of DTT in storage buffer was particularly effective at both maintaining and restoring enzyme activity and this is attributed to reduction of oxidized thiol groups on the enzymes (*14*). The immobilized enzymes did not tolerate repeated freezing at −20°C and thawing. However, if the freezing was carried out rapidly in liquid nitrogen then immobilized enzymes in a glycerol containing storage buffer were stable to repeated freezing/thawing and prolonged storage. Despite the inadvisability (for chromatographic purposes) of freezing Sepharose (*15*), it seems that the Sepharose-immobilized enzymes can be stored frozen for prolonged periods of time without any detrimental effect on activity.

The activities of immobilized enzymes are sensitive to the physical and chemical environment in their immediate vicinity—the "microenvironment." Other authors (*16*) have shown that the introduction of positively charged groups shifts pH optima to lower values, whilst negatively charged groups have the opposite effect. The ability to manipulate th pH optima of immobilized enzymes would be particularly useful when co-immobilizing several enzymes with differing pH optima. Attempts to influence the pH optima of the coimmobilized oxidoreductase/bacterial luciferase or immobilized firefly luciferase using blocking groups with different charges was not successful. Although for the former, the shape of the pH-activity profiles did differ depending on the blockers (Fig. 4). This may be a result of the low concentrations of these blockers on the Sepharose. In contrast to the pH-activity profiles, stability of immobilized enzymes was influenced by the nature of the blocking group. Positively charged ethylenediamine blockers were most effective. This may result from a higher concentration of DTT anions in the microenvironment because of electrostatic interaction with the positively charged Sepharose-bound ethylenediamine groups.

Conclusion

The bioluminescent NADH : FMN oxidoreductase/bacterial luciferase-coupled enzyme system and firefly luciferase can be reproducibly immobilized onto Sepharose 4B and CL 6B. The immobilized enzymes provide a sensitive and reproducible method for detecting NADH or ATP. Both are subject to analytical interferences from proteins, and for the bacterial luciferase system, dehydrogenase enzymes also interfere in NADH assays. However, dilution and/or heat treatment of specimens minimize these interferences. The good stability of the different immobilized enzyme preparations is particularly important since likely application of immobilized bacterial luciferase/oxidoreductase and firefly luciferase will be in the detection of NADH and ATP generated by other co-immobilized enzymes. Thus the stability of such coimmobilized multienzyme systems will not be critically dependent upon the stability of the bioluminescent enzymes.

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