Communications

Stabilization of a Formate Dehydrogenase by Covalent Immobilization on Highly Activated Glyoxyl-Agarose Supports

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Received December 13, 2005; Revised Manuscript Received January 20, 2006

Formate dehydrogenase (FDH) is a stable enzyme that may be readily inactivated by the interaction with hydrophobic interfaces (e.g., due to strong stirring). This may be avoided by immobilizing the enzyme on a porous support by any technique. Thus, even if the enzyme is going to be used in an ultra-membrane reactor, the immobilization presents some advantages. Immobilization on supports activated with bromocianogen, polyethylenimine, glutaraldehyde, etc., did not promote any stabilization of the enzyme under thermal inactivation. However, the immobilization of FDH on highly activated glyoxyl agarose has permitted increasing the enzyme stability against any distorting agent: pH, *T*, organic solvent, etc. The time of support-enzyme reaction, the temperature of immobilization, and the activation of the support need to be optimized to get the optimal stability-activity properties. Optimized biocatalyst retained 50% of the offered activity and became 50 times more stable at high temperature and neutral pH. Moreover, the quaternary structure of this dimeric enzyme becomes stabilized by immobilization under optimized conditions. Thus, at acidic pH (conditions where the subunit dissociation is the first step in the enzyme inactivation), the immobilization of both subunits of the enzyme on glyoxyl-agarose has allowed the enzyme to be stabilized by hundreds of times. Moreover, the optimal temperature of the enzyme has been increased (even by 10 °C at pH 4.5). Very interestingly, the activity with NAD^+-dextran was around 60% of that observed with free cofactor.

Introduction

One of the main targets of modem chemistry is the fully specific asymmetric synthesis of chiral compounds. In this sense, the use of dehydrogenases as asymmetric catalysts may be one of the best alternatives supplied by nature.[1−8] Dehydrogenases tend to be fully selective (e.g., alcohol deshydrogenase from yeast commits only one "error" per 7×10^9 reaction cycles),[9] and the rate of the nonenzymatic process virtually equals zero. Therefore, dehydrogenases are promising alternative catalysts for the production of optically active compounds of extremely high optical purity.

However, these enzymes require the utilization in stoichiometric amounts of an expensive cofactor (NADH or NADPH).[1−8] This hindered the implementation of the biocatalytic processes using dehydrogenases. To shortcut this problem, the regeneration of the cofactor is necessary. NADH recycling is a quite challenging task, where the enzymatic means are the most promising.[10]

Among the candidates for this regeneration of NADH, formate dehydrogenase (FDH) has been extensively studied.[5−8] The advantages of this enzyme are many: a favorable thermodynamic equilibrium, the inertness of the reaction product, (CO_2), and its ready removal from the main product (the product is CO_2 that will go to the atmosphere). Moreover, formate (the substrate) is cheap and also quite inert.

To fulfill this task, FDH should be very stable. Thus, some efforts have been performed on the stabilization of the enzyme by genetic methods.[5−8,11−13]

The fact that in many instances the enzyme is used in ultrafiltration reactors, using cofactors attached to a polymer, promotes that enzyme recovery is not a problem. Due to this reason there have been few attempts to prepare immobilized biocatalysts for this enzyme.[14−17] However, many of the reasons to immobilize any enzyme for its industrial use are still valid in this case: enzymes immobilized inside porous supports may be stabilized against any intermolecular process (e.g., CO_2 bubbles produced during reaction, gas bubbles induced by strong stirring necessary to maintain pH and eliminate CO_2, etc.), it is possible to use very high concentrations of enzymes without risk of precipitation, it is possible to simplify the reactor design, etc.

The use of preimmobilized proteins in membrane reactors even adds some specific advantages to the previously described ones such as preventing the enzyme adsorption on the membrane, increasing the lifetime of the membrane. Obviously, advantages will be much more evident if the enzyme is significantly stabilized due to the immobilization, e.g., via

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10.1021/bm050947z CCC: $33.50
Published on Web 02/10/2006

multipoint or multisubunit covalent attachment.[18−20] To achieve this goal, it is necessary to carefully choose the immobilization system (i.e., support and immobilization conditions[18−20]).

Among the different strategies available and supports to stabilize enzymes by multipoint covalent attachment, glyoxyl agarose may be one of the most effective because of its features, being one of the most relevant ones that it is able to direct the enzyme immobilization by the areas that are the richest in primary amino groups, the reactive groups.[21−23] This supports has been utilized to highly stabilize many different enzymes with high activity recovery values.[23]

Cross-linking with glutaraldehyde of adsorbed enzymes and aminated supports has also been described as a simple way to obtain an intense support−enzyme multipoint attachment.[24] Requirement for a successful result of this strategy is that the enzyme may withstand the chemical modification with glutaraldehyde.

In this manuscript, we have intended to prepare an immobilized and highly stabilized biocatalyst of FDH from *Pseudomonas sp 101* using the methods described above. This enzyme structure has been resolved, being a homo dimer with a molecular weigh of 80 000 Da, which does not contain metal ions or other prosthetic groups in the active center.[25−26] The enzyme has been used in many instances,[5−8,13,27−31] mainly in its soluble form.

Materials and Methods

1. Materials. Formate dehydrogenase (FDH) from *Pseudomonas sp. 101* and nicotinamide adenine dinucleotide (NAD^+) were purchased from Jülich Fine Chemicals. Cyanogen bromide 4 B Sepharose was obtained from Pharmacia Fine Chemicals (Upsala, Sweden). Cross-linked agarose beads (4, 6, and 10%) were from Iberagar S. A. (Portugal). 25 KDa polyethylenimine (PEI), 40 KDa dextran-NAD^+, and formic acid were supplied by Sigma-Aldrich Chem. Co. All supports were prepared as previously described: glyoxyl agarose,[32] MANAE-agarose,[33] and PEI-agarose.[34] All other used reagents were of analytical grade.

2. Methods.

2.1. Preparation of Formate Dehydrogenase. Commercial preparation of FDH was diluted (3/100) in 5 mM sodium phosphate at pH 7.0 and then dialyzed thrice against 50 volumes of 5 mM sodium phosphate at pH 7.0. 100% of initial activity was recovered after this process. The final solution had 0.8 protein mg/mL. The protein concentration was determined using Bradford's method,[35] using BSA as standard.

2.2. Enzymatic Activity Assays. The activity of the different FDH preparations was analyzed spectrophotometrically recording the increment of absorbance at 340 nm promoted by the formation of NADH during the oxidation of formic acid. A sample of enzymatic preparation (25−400 μL) was added to a cell containing 2 mL of 100 mM formic acid and 100 μL of 100 mM NAD^+ in 100 mM sodium phosphate at pH 7.0 and 25 °C. When indicated, different temperatures and pHs were used. In some instances, an equivalent amount of dextran-NAD^+ was used instead of the free cofactor.

One FDH unit (U) was defined as the amount of enzyme necessary to oxidize 1 μmol of formic acid per minute at pH 7 and 25 °C. Soluble FDH prepared as described above had 2.5 U/mL.

2.3. Immobilization of the Enzyme. An enzyme solution at the indicated pH and conditions was mixed with a given amount of the different supports. At different times, samples of supernatants, suspensions, and enzyme solutions under identical conditions but incubated in the presence of inert support were taken and the activity and/or the protein concentration were assayed. All of the experiments were performed using 1 U per mL of support in order to avoid diffusion problems that could alter the operational stability.

2.3.1. Immobilization on CNBr Activated Sepharose 4 B. The immobilization was carried out adding two grams of support to 10 mL of soluble FDH prepared as previously described in 88 mL of 100 mM sodium phosphate at pH 7. The suspension was left under mild stirring during 10 min at 4 °C. After this time the support was filtered and washed with 100 mM sodium bicarbonate at pH 8.3 and incubated (for 2 h) in 1 M ethanolamine at pH 8 to block the remaining CNBr groups. Finally, the immobilized preparation was washed with distilled water.

2.3.2. Immobilization on MANAE-Agarose or PEI-Agarose. The immobilization was carried out adding 3 g of support to 17 mL of 5 mM sodium phosphate pH 7 containing 2.25 mL of soluble FDH prepared as described above. The suspension was gently stirred at 25 °C. Finally, the immobilized FDH was washed with distilled water.

MANAE and PEI agarose derivatives cross-linked with glutaraldehyde were prepared adding 0.5% V/V glutaraldehyde to a suspension (0.1 g/mL) of derivative in 5mM sodium phosphate. The suspension was gently stirred at 25 °C for 30 min. The final derivative was washed with an excess of distilled water.

2.3.3. Immobilization on Sepabeads-Epoxy Supports. The immobilization was carried out adding 3 g of support to 17 mL of 1 M sodium phosphate pH 7 containing 2.25 mL of soluble FDH prepared as described above. The suspension was gently stirred at 25 °C. Periodically, samples of supernatant and suspension were taken, and the activity was checked. After 3 h, the immobilized preparations were washed and suspended in 100 mM sodium bicarbonate pH 10. After 2 h, this preparation was washed and suspended in 20 mL of 3M glycine overnight. Finally the immobilized derivative was washed with distilled water.

2.3.4. Immobilization on Glyoxyl-Agarose. The immobilization was carried out adding three grams of support to 17 mL of 100mM sodium bicarbonate pH 10.05 containing 2.25 mL of soluble FDH prepared as previously described. The suspension was gently stirred at 4 °C.

In some instances, after enzyme immobilization, the suspension was incubated at 25° C to permit a more intense multipoint covalent attachment. Finally, the immobilized preparations were reduced for 1 h at 4 °C by adding 10 mL of 100 mM sodium bicarbonate pH 10 containing 1 mg/mL of sodium borohydride.[36] After this time the preparation was washed with an excess of distilled water.

2.4. Inactivation in the Presence of Gas−Liquid Interfaces under Strong Stirring. 500 μL of soluble FDH or 0.5 g of the CNBr preparation was diluted in 5 mL of 25 mM sodium phosphate pH 7.0. The preparations were very vigorously stirred at 1600 rpm. Experiments were carried out at 25 °C. At different times, samples were withdrawn, and the residual activity was measured as described above.

2.5. Inactivation of Different FDH Preparations. The different FDH preparations (soluble or immobilized enzyme) were incubated at different temperatures and pH values, or in the presence of different solvents. Samples were withdrawn at different times, and the residual activity was measured as previously described.

2.6. SDS−Page Analysis of FDH Derivatives. Samples of soluble and FDH derivatives were analyzed by SDS−PAGE.[37] The enzyme preparations were boiled in the presence of mercaptoethanol and SDS. This treatment releases from the support any protein molecule that was not covalently bound (directly or indirectly) to the support.[38] Samples were injected in a SE 250-Mighty Small II electrophoretic unit (Hoefer Co.) using gels of 12% polyacrylamide in a separation zone of 9 × 6 cm and a concentration zone of 5% polyacrylamide. The gels were stained following the Comassie method. Molecular weight markers were from the LMW kit (14 400−94 000) from Pharmacia.

2.7. Influence of pH and Temperature on the Activity of Immobilized FDH. The influence of pH value on the enzyme activity was analyzed measuring the activity of soluble FDH and FDH immobilized onto glyoxyl-agarose with 100 mM formic acid prepared in different buffers: 100 mM sodium acetate pHs 4 and 5, 100 mM sodium phosphate pHs 6 and 7, 100 mM sodium borate pH 8, 100

Table 1. Immobilization Yield and Expressed Activity of Different FDH Derivatives

derivatives	immobilization yield	expressed activity
MANAE agarose	100%	100%
PEI agarose	100%	100%
MANAE + GLU (0.5%)	100%	17%
MANAE + GLU (0.1%)	100%	80%
MANAE-GLU	100%	10%
Sepabeads	100%	30%
GA 10 BCL 1.5 h	100%	80%
GA 10 BCL 60 min 25 °C	100%	55%
GA 10 BCL 24 h 25 °C	100%	20%

mM sodium bicarbonate pHs 9 and 10. The effect of temperature was studied measuring the activity of soluble FDH and FDH immobilized onto glyoxyl-agarose with 100 mM formic acid prepared in 100 mM sodium acetate pH 4.5 at different temperatures. The activity was measured during the first 180 s of reaction.

Results

Stability of Soluble Enzyme. Effect of Random Immobilization. The FDH from *Pseudomonas sp 101* is a quite stable enzyme at pH 7, remaining fully active after 10 h at 50 °C. Even at 63 °C and pH 7, the half-life of the soluble enzyme is around 1 h. Immobilization of the enzyme using CNBr-agarose did not promote any significant effect on the stability of the enzyme, maintaining a half-life close to 1 h at 63 °C and pH 7. That is, random immobilization is not able to promote a significant increase on the enzyme rigidity. However, the immobilization of an enzyme inside the porous structure of a solid may have some stabilizing effects because it prevents some intermolecule or protein−surface interactions. Thus, under strong stirring (e.g., necessary to eliminate the CO_2 bubbles and keep the pH value), diluted soluble enzyme became rapidly inactivated (just in some minutes) even at 25 °C, whereas CNBr-agarose-FDH remained fully active for long time periods.

From these results, we decided to use the CNBr-agarose-FDH preparations as a reference to compare the stabilization achieved by using other immobilization methodologies.

Immobilization of FDH on Different Supports. To improve the enzyme stability, we have tried different immobilization techniques: reversible immobilization by ionic adsorption on anionic exchangers such as MANAE and PEI-agarose (that could be further cross linked with glutaraldehyde) and glyoxyl agarose (Table 1).

The immobilization on ionic exchangers permitted the recovery of full activity. However, the treatment of the enzyme with glutaraldehyde (0.5% V/V glutaraldehyde) of the already adsorbed protein promoted a drop in enzyme activity; residual activity was around 20%. Glyoxyl agarose immobilized enzyme prepared at 4 °C for 1.5 h exhibited an 80% of the initial activity.

When the stability of the different preparations was compared, only the enzyme immobilized on glyoxyl-agarose was clearly more stable than CNBr-agarose-FDH preparation (Figure 1).

Thus, we tried to further improve the stability of this preparation.

Optimization of Glyoxyl-Agarose-FDH. We studied the effect of the immobilization time, immobilization temperature, and support activation on the features of the immobilized enzyme on glyoxyl agarose. Immobilization was completed after only 1.5 h at 4 °C, and that means that the enzyme has been immobilized by, at least, 2 points.[21−23] However, if the immobilized enzyme is left in contact with the activated support, it is possible that new enzyme-support bonds may be established and this should promote an increase in the enzyme stability. The incubation of the soluble enzyme at 25 °C and pH 10 promoted the rapid enzyme inactivation; thus to study the effect of the temperature in the enzyme stability, we decided to use the enzyme previously immobilized at 4 °C, increasing the temperature to 25 °C just after the enzyme immobilization. Enzyme activity decreased stepwise during the incubation, to a value of 20% after 24 h. The activity recovery after reduction step may be seen in Table 1.

However, enzyme stability seemed not to be strongly dependent on the incubation time, and a slight increase was observed after 1 h of incubation, but stability decreased if incubation time was prolonged to 24 h (Figure 2). Due to the multimeric nature of this enzyme, it is possible that a too intense multi-interaction with the support may alter the subunits

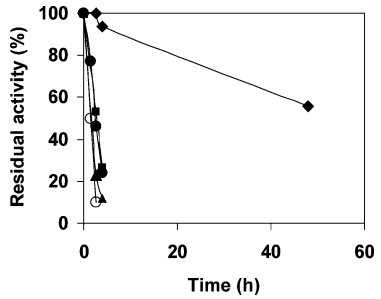

Figure 1. Thermal inactivation courses of different FDH derivatives. (◆) Glyoxyl-agarose 10BCL; (▲) Agarose-PEI 10BCL; (■) CNBr derivative; (●) MANAE-agarose derivative; (○) MANAE-agarose cross-linked with glutaraldehyde. Experiments were carried out at 63 °C in 50 mM sodium phosphate pH 7.

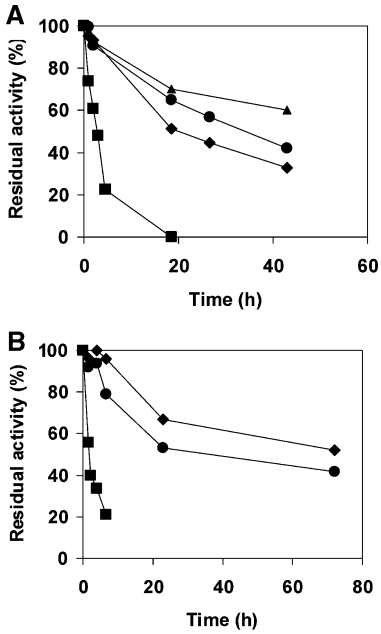

Figure 2. Optimization of glyoxyl FDH preparation. A. Thermal inactivation courses of different glyoxyl-agarose derivatives, influence of time of incubation at room temperature after immobilization. (●) 0 min; (▲) 60 min.; (◆) 16 h; (■) CNBr derivative. Experiments were carried out at 63 °C and in 50 mM sodium phosphate pH 7. B. Influence of the density of reactive groups on glyoxyl-agarose 10BCL in thermal inactivation courses of different glyoxyl-agarose derivatives. (◆) 200 μmol/mL; (●) 100 μmol/mL; (■) 15 μmol/mL. Experiments were carried out at 63 °C in 50 mM sodium phosphate pH 7.

Table 2. Characterization of the Optimal FDH Glyoxyl-Agarose Preparation

conditions	stabilization factor
pH 4; 30 °C	>300
pH 7;63 °C	>50
pH 10; °C, 45 °C	>15
pH 8.5, 25 °C; 75% acetone	>75

assembly, reducing the enzyme stability even though each monomer can be more "rigid".

The decrease in the density of glyoxyl groups on the support surface promoted a decrease in the stabilization achieved; using a support activated with 15 μmol/g, the FDH stability became close to that of the CNBr-agarose-FDH, a decrease of 50% of the glyoxyl groups decreased the enzyme stability by a 2-fold factor (Figure 2).

Thus, as optimal protocol, immobilization of the enzyme at 4 °C in 200 μmol/mL glyoxyl-agarose for 1.5 h was proposed, and then the immobilized preparation was incubated at 25 °C for 1 h and reduced using 1 mg/mL of sodium borohydride.

This derivative was over 50-fold more stable at pH 7 and 63 °C than the CNBr-agarose-FDH preparation (Table 2).

Characterization of the Optimal FDH Glyoxyl-Agarose Preparation. The optimal immobilized enzyme preparation was compared to the CNBr-agarose preparation on different conditions (Table 2). Resistance to organic solvents was very significant for all immobilized preparations, remaining fully active after 24 h in 50% acetone, Dioxane, etc. At 75% acetone, the enzyme immobilized on CNBr-agarose presented a half-life of 5 h, whereas the optimal derivative kept more than 65% initial activity after 4 days.

At pH 4.5, the enzyme stability was greatly reduced. However, the effect of the enzyme immobilization was even more significant on the enzyme stability. Figure 3 shows that the optimal derivative becomes hundreds of times more stable than the CNBr-agarose under these conditions. This could be derived from a higher tendency to dissociation of the enzyme subunits under these conditions. In fact, although at pH 7 an effect of CNBr-agarose-FDH or soluble enzyme concentrations on its stability could not be detected, at pH 4, this effect was quite clear (Figure 3). In fact, the optimal derivative was able to fully stabilize the multimeric structure of the enzyme (no enzyme subunits could be visualized in SDS−PAGE experiments using this derivative but the BrCN preparation released some enzyme subunits) and then a dependence of enzyme stability on enzyme concentration could not be detected (see Figure 3).

The pH/activity profile remained almost unaltered by the immobilization, with a maximum activity at pH 7. However, the activity/temperature profile at pH 4.5 (Figure 4) showed an increase in the optimal temperature of around 10°C, with a residual activity of 65% of this maximum at 65 °C, whereas soluble enzyme was fully inactive under these conditions. At pH 7, the optimal *T* increased at 65 °C for soluble enzyme, and the immobilized preparation permitted the increase of this optimal *T* to 68 °C, being lower the effect of the enzyme stabilization in this case.

Very interestingly, the immobilized enzyme exhibited around 60% of the activity when using dextran-NAD^+. This lower activity could be related to some diffusion problems due to the large size of the polymeric cofactor, in fact, the mechanical breaking of the particles permitted to reach higher activity. Thus, this derivative could be industrially used on ultra-filtration reactors to catalyze the recycling of the NAD^+.

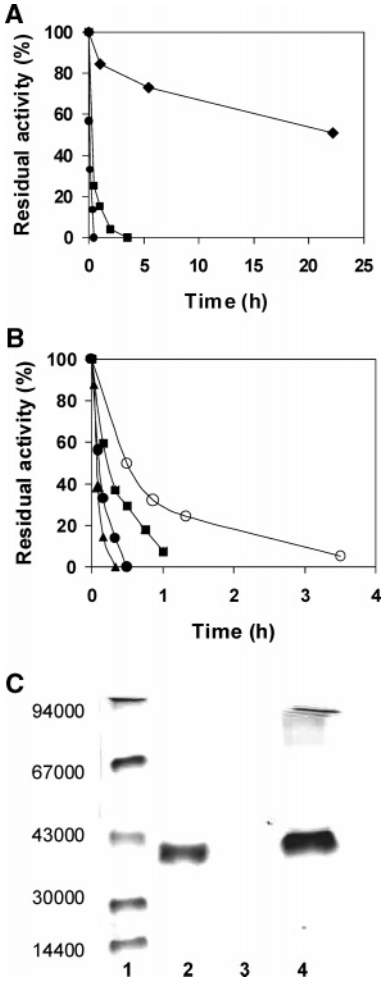

Figure 3. Stability of different FDH preparations under dissociation conditions. (A) Inactivation course of different preparations of 0.2 U/mL FDH: (◆) Glyoxyl-Agarose 10BCL derivative; (■) CNBr derivative; (●) Soluble FDH, 0.2 U/mL Experiments were carried at 30 °C in 50 mM sodium acetate pH 4. (B) Inactivation courses of soluble FDH at different concentrations: (▲) 0.01U/mL; (●) 0.1U/mL; (■) 0.667 U/mL; (○) 1 U/mL Experiments were carried out at 30 °C in 50 mM sodium acetate pH 4. (C) SDS−PAGE analyses of the proteins released from different FDH preparations. Lane 1: Molecular weight markers; lane 2: soluble FDH; lane 3: glyoxyl-agarose derivative; lane 4: CNBr derivative.

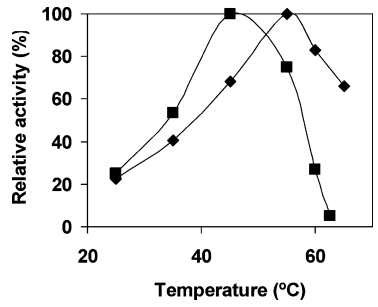

Figure 4. Influence of temperature on the enzymatic activity of different FDH preparations. (■) Soluble FDH. (◆) Glyoxyl-agarose 10BCL. Activity assays were performed in 100 mM formic acid in 100 mM sodium acetate at pH 4.5 and different temperatures. The relative activity was calculated taking 100% the maximum observed activity as described in Methods.

Conclusion

In this manuscript, we describe the preparation of a highly stabilized immobilized biocatalyst of the very relevant enzyme FDH. To achieve optimal activity/stability properties of the

biocatalyst, the selection of glyoxyl-agarose as support to immobilize the enzyme, the density of glyoxyl groups in the support, the temperature and time of immobilization was very important. The final preparation presented stabilized its multimeric structure, making it suitable to be used under a broad range of pH values and in the presence of moderately high concentrations of organic cosolvents, with a 50% of expressed activity. Moreover, the immobilized FDH is able to recognize the cofactor bound to dextran, suggesting a proper orientation of the enzyme that left the active center oriented toward the medium. These features make this immobilization protocol a suitable one to produce an industrial biocatalyst to recycle NAD+ under a wide range of reaction conditions.

Acknowledgment. Authors are thankful for the financial support from Spanish CICYT (project PPQ2005-2420) and CAM (PPQ-344-505), a Ramon y Cajal contract for Dr. Mateo (MEC), a pre-doctoral fellowship from CAM for Mr. Bolivar. We thank Fundación Carolina (Spain) and Ministerio de Educación, Ciencia y Tecnología (Argentine) for a fellowship granted to Dr. Ferrarotti. We thank CONICYT-BID (Chile) for a fellowship for Dr. Wilson. We gratefully recognize the support given by the Program of International Cooperation CSIC (Spain) − CONICYT (Chile). The suggestions from Dr. Ángel Berenguer during the writing of the manuscript are also gratefully recognized.

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BM050947Z