Coating of Soluble and Immobilized Enzymes with Ionic Polymers: Full Stabilization of the Quaternary Structure of Multimeric Enzymes

ARTICLE *in* BIOMACROMOLECULES · APRIL 2009

Impact Factor: 5.75 · DOI: 10.1021/bm801162e · Source: PubMed

7 AUTHORS, INCLUDING:

Juan M Bolivar
Graz University of Technology
48 PUBLICATIONS **761** CITATIONS
SEE PROFILE

Javier Rocha Martín
Abengoa
23 PUBLICATIONS **337** CITATIONS
SEE PROFILE

Felipe Cava
Umeå University
54 PUBLICATIONS **1,092** CITATIONS
SEE PROFILE

José Berenguer
Universidad Autónoma de Madrid
55 PUBLICATIONS **920** CITATIONS
SEE PROFILE

Coating of Soluble and Immobilized Enzymes with Ionic Polymers: Full Stabilization of the Quaternary Structure of Multimeric Enzymes

Juan M. Bolivar,[†] Javier Rocha-Martin,[†] Cesar Mateo,[†] Felipe Cava,[‡] Jose Berenguer,[‡] Roberto Fernandez-Lafuente,*[,†] and Jose M. Guisan*[,†]

Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica-CSIC, Campus UAM, Cantoblanco, 28049 Madrid, Spain, and Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Campus UAM, Cantoblanco, 28049 Madrid, Spain

Received October 13, 2008; Revised Manuscript Received January 28, 2009

This paper shows a simple and effective way to avoid the dissociation of multimeric enzymes by coating their surface with a large cationic polymer (e.g., polyethylenimine (PEI)) by ionic exchange. As model enzymes, glutamate dehydrogenase (GDH) from *Thermus thermophilus* and formate dehydrogenase (FDH) from *Pseudomonas* sp. were used. Both enzymes are very unstable at acidic pH values due to the rapid dissociation of their subunits (half-life of diluted preparations is few minutes at pH 4 and 25 °C). GDH and FDH were incubated in the presence of PEI yielding an enzyme−PEI composite with full activity. To stabilize the enzyme−polymer composite, a treatment with glutaraldehyde was required. These enzyme−PEI composites can be crosslinked with glutaraldehyde by immobilizing previously the composite onto a weak cationic exchanger. The soluble GDH-PEI composite was much more stable than unmodified GDH at pH 4 and 30 °C (retaining over 90% activity after 24 h incubation) with no effect of the GDH concentration in the inactivation course. The composite could be very strongly, but reversibly, adsorbed on cationic exchangers. Similarly, FDH could be treated with PEI and glutaraldehyde after adsorption on cationic exchangers, This permitted a stabilized FDH preparation. In this way, the coating of the enzymes surfaces with PEI is used as a simple and efficient strategy to prevent enzyme dissociation of multimeric enzymes. These composites can be used as a soluble catalyst or reversibly immobilized onto a cationic exchanger (e.g., CM-agarose).

Introduction

The preparation of stable enzyme derivatives is a requisite for the applied use of these highly interesting biocatalysts.[1,2] Fortunately, there are many strategies for enzyme stabilization in rapid development (protein engineering and directed evolution,[3−5] screening of enzymes from thermophilic origin,[6,7] chemical modification,[8] and enzyme immobilization[9,10]) that may even be used in a joint way.[11,12]

An especially complex problem is the stabilization of multimeric enzymes. The first step in the inactivation of these biocatalysts is, in many instances, the dissociation of the enzyme subunits or their wrong assembly.[13−15] For this reason, the prevention of enzyme dissociation tends to be the first target when trying to stabilize these interesting but complex enzymes.[16] There are successful reports on the stabilization of multimeric enzymes by chemical crosslinking,[17−19] enzyme immobilization,[11,20−23] or protein engineering.[24,25]

In this paper, an alternative route for the stabilization of the quaternary structure of multimeric enzymes by simple coating of their surface with large ionic polymers is proposed.

Polyethylenimine (PEI) has been used in some instances to stabilize proteins in solution, by preventing oxidation, aggregation, and so on.[26−28] PEI is a polymer that has a high density of ionized tertiary, secondary, and primary amino groups. In fact, it has been reported that most proteins may become strongly adsorbed on supports coated with PEI at neutral pH value.[29,30] The high density of cationic groups in this large polymer may permit the strong ionic exchange of the polymer on any area of the protein surface having anionic groups, coating its surface. When a multimeric protein is used, due to the availability of PEI with very different sizes, it may be quite likely to find a polymer with a size able to interact with areas on the protein surface located in two (or even several) different enzyme subunits. Thus, the simple incubation of a multimeric enzyme in the presence of PEI could prevent subunit dissociation. This PEI-coated enzyme, perhaps with the quaternary structure already stabilized, could be used in soluble form. Moreover, benefitting from the high amount of cationic groups placed in the polymer, this composite could be strongly but reversibly adsorbed on cationic exchangers (without really involving the protein core in the adsorption). After enzyme inactivation, using drastic enough conditions, the enzyme could be desorbed from the support and reused in new immobilization cycles.

Another possibility to extend the range of conditions where PEI-coated enzymes could be used would be the further covalent crosslinking of the polymer and the enzyme, for example, with glutaraldehyde,[31−33] to give a PEI−enzyme composite completely stable under any experimental condition. This enzyme coated with PEI and crosslinked with glutaraldehyde could still be reversibly but strongly adsorbed on cationic exchangers. In fact, the enzyme−PEI composite could be adsorbed on a cationic exchanger and then treated with glutaraldehyde to take advantage of the solid phase modification of the protein.[34]

* To whom correspondence should be addressed. Phone: 91-585-4809. Fax: 34-91-5854760. E-mail: rfl@icp.csic.es (R.F.-L.); jmguisan@icp.csic.es (J.M.G.).
[†] Instituto de Catálisis y Petroleoquímica-CSIC.
[‡] Centro de Biología Molecular Severo Ochoa.

10.1021/bm801162e CCC: $40.75
Published on Web 03/06/2009

To check the feasibility of these new strategies, two multimeric dehydrogenases have been chosen as model enzymes. The first enzyme is a trimeric glutamate dehydrogenase (GDH) from *Thermus thermophilus*.[35] The second enzyme is a dimeric formate dehydrogenase (FDH) from *Pseudomonas* sp.[36,37]

Both enzymes may be used for the regeneration of NAD(P)H (GDH may also be used to regenerate $NAD(P)^+$). This regeneration of cofactors is a critical requirement for the industrial implementation of dehydrogenases, which are very interesting enzymes for the asymmetric reduction of ketones to the corresponding alcohols.[38,39] GDHs have been also used as biosensors of different compounds.[40,41]

The FDH and GDH that are used in this paper are readily inactivated at acidic pH values by subunit dissociation and the immobilization of all the enzyme subunits on a support have been shown to prevent this problem.[35,42] Dehydrogenases may be applied by using the soluble enzyme trapped in ultrafiltration membrane reactors[38,39] or in the tip of the sensor,[40,41] although their use in a preimmobilized form may have some advantages.[42,43]

Coating of the enzyme surface with a polymer may permit some additional positive effects together with the prevention of the enzyme inactivation by subunit dissociation. On the one hand, the increase in size of the enzyme may simplify the retention of the enzyme on a membrane or sol−gel system.[43−45] On the other hand, the coating of enzyme surfaces with polymers has been described as an efficient method to prevent negative interactions of enzymes and interfaces (e.g., gas bubbles) that may produce enzyme inactivation.[46,47]

Materials and Methods

Materials. Formate dehydrogenase (FDH) from *Pseudomonas* sp. 101 and nicotinamide adenine dinucleotide (NAD^+) were purchased from Jülich Fine Chemicals. Carboxymethyl (CM) agarose beads (4%) were from Amersham Biosciences. Glutamic acid, aspartic acid, formic acid, alpha-keto glutaric acid and polyethyleneimine (MW: 25 KD) were supplied by Sigma-Aldrich Chem. Co. 2,4,6-Trinitrobenzensulfonic acid (TNBS) was from Fluka. Glyoxyl agarose beads (4%) activated with only 1 μmol/g of support were prepared as described elsewhere.[48] All other reagents employed in this study were of analytical grade. Glutamate dehydrogenase from *Thermus thermophilus*, overexpressed in *Escherichia coli*, was produced as published elsewhere and diluted with 5 mM sodium phosphate.[35] Protein concentration was determined using Bradford's method.[49]

Methods. *Preparation of Formate Dehydrogenase Solution.* A commercial preparation of FDH was diluted (3/100) in 5 mM sodium phosphate at pH 7.0 and 4 °C and then dialyzed three times against 50 volumes of 5 mM sodium phosphate at pH 7.0 at 4 °C. A total of 100% of the initial activity was recovered after this process. The final FDH solution had 0.8 mg/mL protein.

Enzyme Activity Assays. GDH Activity Determination. The activities of the different GDH preparations were analyzed by the increase in absorbance at 340 nm, corresponding to the formation of NADH concomitant to L-glutamate oxidation ($\varepsilon = 6.22\ mM^{-1}$ at 340 nm). A sample of enzyme preparation (25−400 μL) was added to a cell with 2 mL of 250 mM glutamic acid in 100 mM sodium phosphate at pH 8.0 and 66 °C. The reaction was started by the addition of 100 μL of 100 mM NAD^+. One GDH unit (U) was defined as the amount of enzyme required to oxidize 1 μmol of glutamic acid per minute at pH 8 and 66 °C. The glutamate dehydrogenase preparation used for the assays had a specific activity of 2.45 U/mg of protein and 1.5 U/mL.

FDH Activity Determination. The activities of the different FDH preparations were 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. 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. FDH solution prepared as described above had 2.5 U/mL.

Coating of the Enzyme Surface with PEI. A total of 4 mL of enzymic solution (1 mg/mL) was mixed with 16 mL of a 25 mg/mL PEI solution at pH 7 and 4 °C. After 16 h, the solution was dialyzed against 5 mM sodium phosphate at pH 7 using a membrane with a size-cut of 100 KDa (five changes with a ratio 1/1000 v/v) at 4 °C. The coating was confirmed by titration of primary amino groups with TNBS.[50]

Preparation of Aspartic Agarose. A very low-activated support was prepared in some instances, to facilitate desorption of the PEI−enzyme composite from the support. A total of 10 g of glyoxyl 4% CL agarose beads (having 1 μmol of glyoxyl groups/g of support) was added to 100 mL of 3 M sodium aspartate at pH 10.1 and 25 °C and gently stirred for 2 h. Then, 100 mg of sodium borohydride was added to reduce the imino bonds,[51] and after 30 min, the suspension was washed with an excess of distilled water using a sintered glass funnel.

Immobilization on CNBr-Activated Sepharose 4 B. The immobilization was carried out as described previously.[35,42]

CNBr Agarose Derivative Coated with PEI. A total of 1 g of GDH or FDH-CNBr derivatives was mixed with 16 mL of a 25 mg/mL PEI solution at pH 7 and 4 °C under mild stirring. After 16 h, the modified derivatives were washed with distilled water.

Adsorption of PEI-Coated Enzymes on CM-Agarose or Aspartic-Agarose. A total of 1 g of cationic exchanger support (CM-agarose or Asp-agarose) was added to 9 mL of a solution of the respective enzyme (0.1 mg/mL) coated with PEI under mild stirring at 25 °C. Periodically, samples of the suspensions and supernatants were withdrawn and their activity determined as described above. The immobilized enzymes were vacuum filtered using a sintered glass funnel and washed with an excess of distilled water.

Modification of the Coated Enzyme with Glutaraldehyde. A total of 1 g of the coated and immobilized enzyme preparation or 1 mL of the soluble coated enzyme solution was added to 9 mL of a solution containing 0.5% glutaraldehyde at pH 7 and 4 °C under mild stirring. After 1 h, the biocatalysts were washed as above or dialyzed against 5 mM sodium phosphate at pH 7.

Desorption of the Enzyme from the Cationic Exchangers. The immobilized enzymes were incubated 1/10 (w/v) in 5 mM sodium phosphate at pH 7 and 25 °C containing increasing concentrations of NaCl under mild stirring. Activities of suspensions and supernatants were determined as described above. In some cases, the desorbed enzyme was dialyzed to leave it in the desired buffer.

Inactivation of Different Enzyme Preparations. The different enzyme preparations (soluble or immobilized enzyme) were incubated at different temperatures and pH values. Samples were withdrawn (200 μL) at different times after stirring and residual activity was measured, as previously described.

Results

Coating of the Soluble Enzymes with PEI. Incubation of GDH or FDH with PEI under the conditions described in the Methods section did not affect the enzyme activity, recovering more than 95% of the initial activity after PEI incubation and PEI−enzyme purification protocol. The titration of primary amino groups with TNBS of the enzymes incubated in the presence of PEI showed an increase in adsorption at 405 nm by factor higher than 5. This suggested that the enzymes have been coated with PEI, very likely by several PEI molecules per enzyme molecule.

Effect of the PEI Coating on the Stability of Soluble and Immobilized Enzymes. Figures 1 and 2 show that both enzymes (after coating with PEI) are rapidly inactivated at pH 4. Moreover, the inactivation rates increased when the enzymes

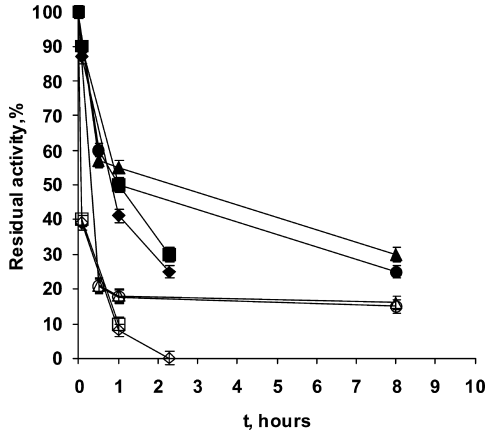

Figure 1. Inactivation curves of different GDH preparation at pH 4. Experiments were carried out in 200 mM sodium acetate at 25 °C. Other specifications are described in Methods. (●) GDH-CNBr (0.6 U /mL); (○) GDH-CNBr (0.1 U/mL); (▲) GDH-CNBr coated with PEI (0.6 U/mLl); (△) GDH-CNBr coated with PEI (0.1 U/mL); (■) soluble GDH (0.6 U/mL); (□) soluble GDH (0.1 U/mL); (◆) soluble GDH coated with PEI (0.6 U/mL); (◇) soluble GDH coated with PEI (0.1 U/mL).

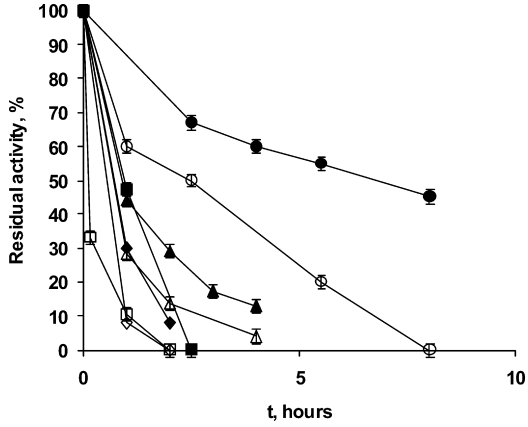

Figure 2. Inactivation curves of different FDH preparation at pH 4. Experiments were carried out in 200 mM sodium acetate at 25 °C. Other specifications are described in Methods. (●) FDH-CNBr (0.6 U /mL); (○) FDH-CNBr (0.1 U/mL); (▲) FDH-CNBr coated with PEI (0.6 U/mL); (△) FDH-CNBr coated with PEI (0.1 U/mL); (■) soluble FDH (0.6 U/mL); (□) soluble FDH (0.1 U/mL); (◆) soluble FDH coated with PEI (0.6 U/mL); (◇) soluble FDH coated with PEI (0.1 U/mL).

concentration decreased. As previously reported,[35,42] the immobilization of both enzymes on CNBr did not prevent this phenomenon, being a good model to check in a simple way if the incubation with PEI could be an efficient way to stabilize the multimeric structure of the enzyme. The incubation with PEI of enzyme−CNBr preparations had no effect on the enzymes stability at pH 4. Thus, the coating of the enzyme with PEI seems to have no effect on the enzyme stability at pH 4. This could be explained by two different reasons: the used PEI is not large enough to "ionically crosslink" all the enzyme subunits of the enzymes or, at this low pH, where the surface of the enzyme will take a positive charge, PEI molecules may be released from the enzyme surface. This was confirmed when the BrCN immobilized enzyme preparations were treated with PEI and then washed at pH 4. While the preparations treated with PEI showed an increase in color developed by incubation with TNBS, the preparation incubated at pH 4 showed a color similar to that of the initial preparation.

This desorption of the PEI may be prevented by treating the enzyme−PEI composite with glutaraldehyde, if it is able to make covalent bonds between the PEI and the enzyme molecules. This should produce a covalent enzyme−polymer composite, and the composite should remain associated under any pH value.

Preparation of PEI−Enzyme Composites Crosslinked with Glutaraldehdye. When the GDH-PEI composite solution was treated with glutaraldehyde, it was possible to observe the formation of large aggregates, very likely due to the production of covalent aggregates by crosslinking between several enzyme−PEI composites.

To overcome this problem, GDH-PEI and FDH-PEI composites were adsorbed on CM-agarose at pH 7, using very low loadings to prevent the formation of protein aggregates (Figure 3). At pH 7, the native enzyme did not immobilize on this support (results not shown). However, immobilization of the enzyme−PEI composites was very rapid. The enzyme composites remained fully active after immobilization. These adsorbed proteins were later treated with glutaraldehyde. Afterward, desorption of the enzymes was intended to recover the soluble PEI−enzyme composite. However, the adsorption of the composites on the support was very strong, making their release from the support very difficult (both enzyme composites remained fully adsorbed on the support even after incubation in 2 M sodium phosphate at pH 7).

Thus, a lowly activated aspartic-agarose support was used to adsorb the PEI-GDH composite. This adsorbed composite could be treated with glutaraldehyde and later fully desorbed from the support by incubation in 1 M sodium phosphate at pH 7 and 25 °C, retaining more than 95% of the initial activity.

Stability of PEI-GDH−Glutaraldehyde Composites under Acidic Conditions. This GDH-PEI crosslinked with glutaraldehyde composite was very stable at pH 4 and 30 °C, and the stability did not depend on the enzyme concentration (Figure 4).

A similar treatment with glutaraldehyde of GDH immobilized on CNBr (preparation that released some subunits and whose stability depended on the enzyme concentration at pH 4) showed that the inactivation courses at pH 4 of these preparations remained identical in shape and dependence with the enzyme concentration when compared to the unmodified CNBr-GDH (Figure 4). This showed that the enzyme subunits of GDH were not directly crosslinked by the glutaraldehyde treatment. Therefore, the effect found for the treatment of the PEI-GDH composite with glutaraldehyde should be due to the crosslinking of the GDH with PEI molecules, which involves several enzyme subunits and avoids the risks of subunit dissociation.

Other Positive Effects of Coating with PEI. Diluted solutions of enzymes could be easily inactivated under strong stirring due to the interaction of the enzymes with the hydrophobic gas bubbles. The coating of the enzyme surface with polymers may avoid this interaction,[46] giving a stable enzyme preparation under strong stirring conditions. Figure 5 shows that GDH was very rapidly inactivated at pH 7 under strong stirring. However, GDH-PEI composites were much more resistant to stirring, suggesting that most of its surface was covered by the hydrophilic polymer.

Preparation of GDH-PEI Immobilized on CM-agarose. Adsorption of the crosslinked composite on CM-agarose permitted to have the enzyme in an immobilized form. The stability and activity of this immobilized enzyme was identical to that of the free composite, but having the advantages of a reversible immobilization concerning handling and reactor design. After enzyme inactivation, the enzyme−PEI composite could be desorbed from the support by incubation in 9 M guanidine

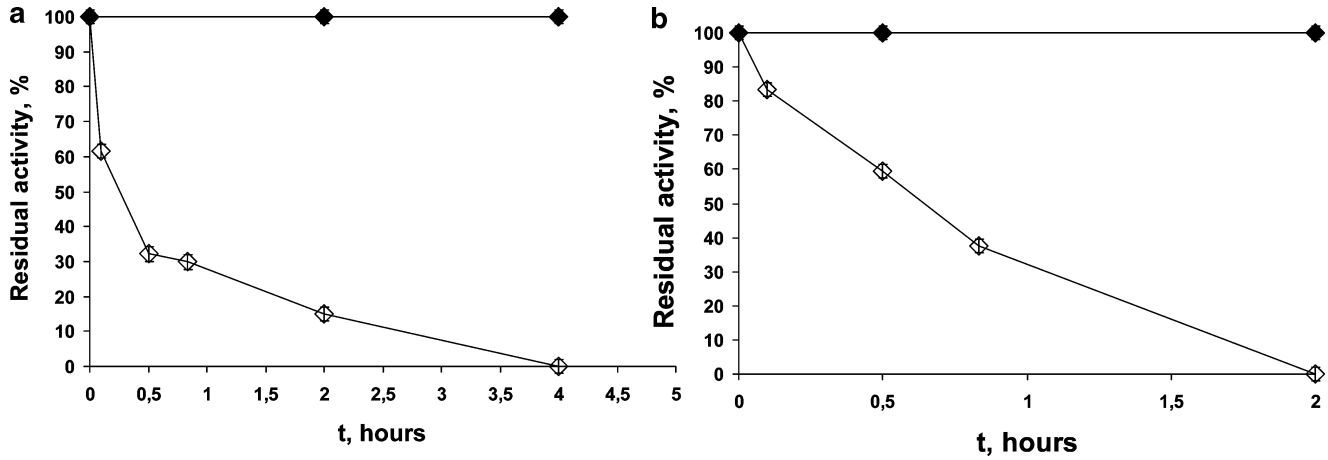

Figure 3. Immobilization course of composites enzyme−PEI onto CM-agarose. Experiments were carried out in 10 mM sodium phosphate at pH 7 and 25 °C as described in Methods. (a) FDH-PEI: (◆) suspension; (◇) supernatant. (b) GDH-PEI: (◆) suspension; (◇) supernatant.

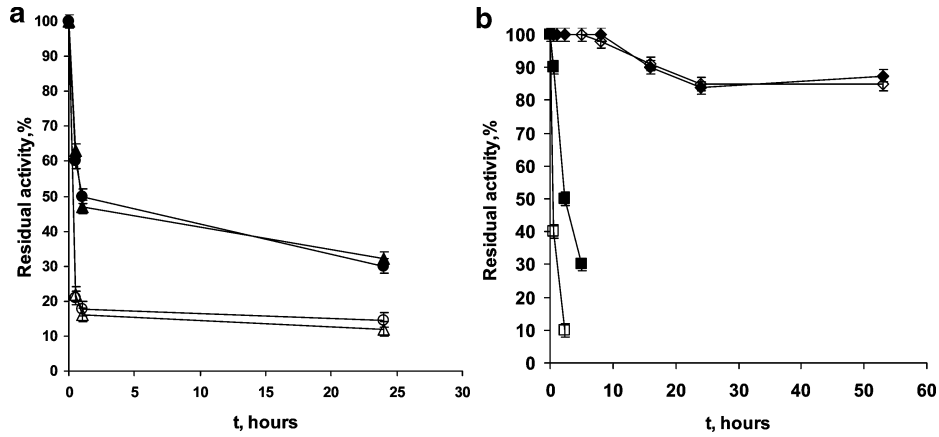

Figure 4. Inactivation curves of different GDH derivatives at pH 4. Experiments were carried out in 200 mM sodium acetate at 25 °C as described in Methods. (a) (●) GDH-CNBr (0.6 U /mL); (○) GDH-CNBr (0.1 U/mL); (▲) GDH-CNBr treated with glutaraldehyde (0.6 U/mL); (△) GDH-CNBr treated with glutaraldehyde (0.1 U/mL). (b) (◆) Soluble GDH-PEI treated with glutaraldehdye (0.6 U/mL); (◇) soluble GDH-PEI treated with glutaraldehdye (0.1 U/mL); (■) soluble GDH (0.6 U/mL); (□) soluble GDH (0.1 U/mL).

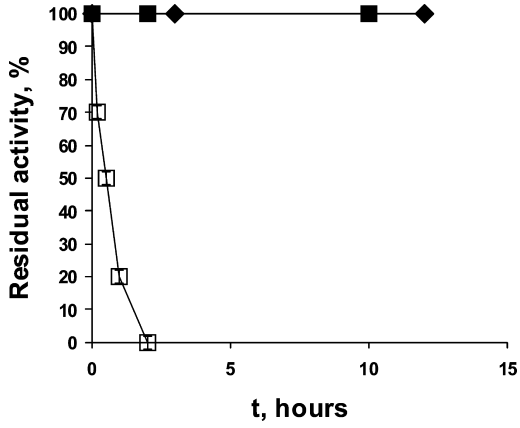

Figure 5. Effect of stirring on the activity of different preparations of GDH. Experiments were carried out in 200 mM sodium phosphate at pH 7 and 4 °C, the enzyme solutions were stirred at 1000 rpm. (■) Nonstirred GDH (0.1 U/ mL); (□) stirred GDH (0.1 U/mL); (◆) stirred PEI-GDH (0.1 U/mL).

chloride at 40 °C (no amine groups could be detected in the support by titration with TNBS after this washing) and the CM-support could be reused for three immobilization/desorption cycles without any change in its properties.

If the enzyme is going to be used in an immobilized form, the treatment of the GDH-PEI composite with glutaraldehyde could be performed directly on the composite adsorbed on CM-agarose, this preparation presented similar stability and activity recovery (results not shown). This greatly simplifies the preparation of this immobilized biocatalyst.

Thus, to have a reversibly immobilized preparation, CM-agarose could be used in the first adsorption, while to have a soluble enzyme composite, it is necessary to use a low-activated cationic exchanger (to desorb the enzyme under mild conditions).

Preparation of a CM-agarose FDH-PEI Immobilized Preparation. FDH-PEI composite was also adsorbed on CM-agarose and then treated with glutaraldehyde. The activity recovery after the whole treatment was 40% (the activity loss was due to the glutaraldehyde treatment). Figure 6 shows that the inactivation rate of the immobilized enzyme composite at pH 4 was similar using different enzyme concentrations, suggesting the prevention of enzyme subunits dissociation as a cause of inactivation. The stability of these preparations was much higher than the stability of FDH immobilized in CNBr (preparation whose stability at pH 4 depends on the concentration and that has only one subunit attached to the enzyme).[42] However, the enzyme can be inactivated at pH 4 just because of conformational changes produced by these drastic pH conditions (this is not a thermophilic enzyme, and the rigidity of each monomer may be not significantly improved).

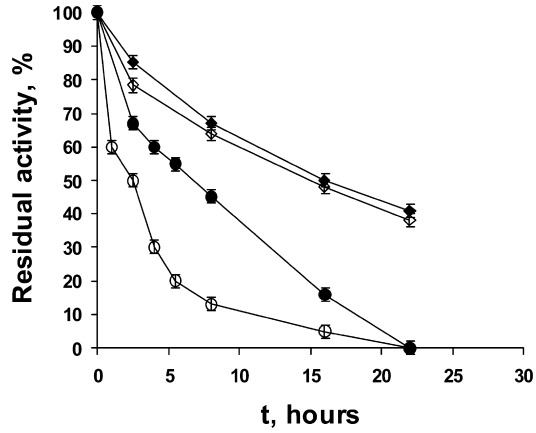

Figure 6. Inactivation curves of different FDH derivatives at acidic pH. Experiments were carried out in 200 mM sodium acetate at 25 °C at pH 4. (●) FDH-CNBr (0.6 U /mL); (○) FDH-CNBr (0.1 U/mL); (◆) FDH-PEI immobilized on CM-agarose (0.6 U/mL); (◇) FDH-PEI immobilized on CM-agarose (0.1 U/mL).

Conclusions

The incubation of enzymes in the presence of PEI seems to be a simple strategy to obtain enzyme-polymers composites that offers some advantages. First, the enzyme coated with PEI may be very strongly adsorbed on cationic exchangers, in a reversible way, but without involving the enzyme core in the adsorption. Second, the soluble enzymes coated with this polymer are stable against the interaction with interfaces (e.g., gas bubbles). And finally, if the enzyme is a multimeric one, like those used in this paper, this may be a simple strategy to prevent dissociation of the enzyme subunits.

Due to the reversible nature of the polymer−enzyme composite, under certain conditions the polymer may be released from the enzyme surface, losing its protective effect. To solve this problem, the PEI−enzyme may be crosslinked with glutaraldehyde. This must be performed in solid phase to prevent enzyme−PEI/enzyme−PEI aggregation (although these crosslinked aggregates could be directly an interesting form of enzyme immobilization).

In the case of the enzymes used in this paper, the treatment has permitted biocatalysts in soluble or (reversible) immobilized form that may not dissociate at acidic pH values, and in that way, their stabilities were greatly improved under these conditions.

Acknowledgment. This work has been supported by Comunidad Autónoma de Madrid (CAM; Grant S0505/PPQ/0344) and MEC (Grants BIO2004-0267 and CTQ2005-02420/PPQ). An institutional grant from Fundación Ramón Areces to CBMSO is also acknowledged. J.M.B. and J.R.-M. are the holders of a Ph.D. fellowship from CAM, and F.C. holds a contract from the MEC. The suggestions from Dr. Angel Berenguer (University of Alicante) and Paul Shadbolt (Cambridge University) during the writing of this paper are gratefully recognized.

References and Notes

(1) Kim, J.; Grate, J. W.; Wang, P. *Chem. Eng. Sci.* **2006**, *61*, 1017–1026.
(2) Polizzi, K. M.; Bommarius, A. S.; Broering, J. M.; Chaparro-Riggers, J. F. *Curr. Opin. Chem. Biol.* **2007**, *11*, 220–225.
(3) Pollegioni, L.; Sacchi, S.; Caldinelli, L.; Boselli, A.; Pilone, M. S.; Piubelli, L.; Molla, G. *Curr. Protein Pept. Sci.* **2007**, *8*, 600–618.
(4) Montiel, C.; Bustos-Jaimes, I. *Curr. Chem. Biol.* **2008**, *2*, 50–59.
(5) Gershenson, A.; Arnold, F. H. *Genet. Eng.* **2000**, *22*, 55–76.
(6) Antranikian, G.; Vorgias, C. E.; Bertoldo, C. *Adv. Biochem. Eng. Biotechnol.* **2005**, *96*, 219–262.
(7) Razvi, A.; Scholtz, J. M. *Protein Sci.* **2006**, *15*, 1569–1578.
(8) O'Fágáin, C. *Enzyme Microb. Technol.* **2003**, *33*, 137–149.
(9) Kim, J.; Grate, J. W.; Wang, P. *Chem. Eng. Sci.* **2006**, *61*, 1017–1026.
(10) Polizzi, K. M.; Bommarius, A. S.; Broering, J. M.; Chaparro-Riggers, J. F. *Curr. Opin. Chem. Biol.* **2007**, *11*, 220–225.
(11) Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2007**, *40*, 1451–1463.
(12) Padma, V. I.; Ananthanarayan, L. *Process Biochem. (Amsterdam, Neth.)* **2008**, *43*, 1019–1032.
(13) Pilipenko, O. S.; Atyaksheva, L. F.; Poltorak, O. M.; Chukhrai, E. S. *Russ. J. Phys. Chem.* **2007**, *81*, 990–994.
(14) Poltorak, O. M.; Chukhray, E. S.; Torshin, I. Y. *Biochemistry (Moscow)* **1998**, *63*, 303–311.
(15) Poltorak, O. M.; Chukhrai, E. S.; Kozlenkov, A. A.; Chaplin, M. F.; Trevan, M. D. *J. Mol. Catal. B: Enzym.* **1999**, *7*, 157–163.
(16) Eijsink, V. G. H.; Bjørk, A.; Gåseidnes, S.; Sirevåg, R.; Synstad, B.; Burg, B. V. D.; Vriend, G. *J. Biotechnol.* **2004**, *113*, 105–120.
(17) Fuentes, M.; Segura, R. L.; Abian, O.; Betancor, L.; Hidalgo, A.; Mateo, C.; Fernandez-Lafuente, R.; Guisan, J. M. *Proteomics* **2004**, *4*, 2602–2607.
(18) Chiu, W.-C.; You, J.-Y.; Liu, J.-S.; Hsu, S.-K.; Hsu, W.-H.; Shih, C.-H.; Hwang, J.-K.; Wang, W. C. *J. Mol. Biol.* **2006**, *359*, 741–753.
(19) Fernandez-Lafuente, R.; Hernandez-Juístiz, O.; Mateo, C.; Terreni, M.; Fernandez-Lorente, G.; Moreno, M. A.; Alonso, J.; Garcia-Lopez, J. L.; Guisan, J. M. *Biomacromolecules* **2001**, *2*, 95–104.
(20) Nahalka, J.; Dib, I.; Nidetzky, B. *Biotechnol. Bioeng.* **2008**, *99*, 251–260.
(21) Vikartovskaí, A.; Bucko, M.; Mislovicôvaí, D.; Paåtoprstyí, V.; Laciík, I.; Gemeiner, P. *Enzyme Microb. Technol.* **2007**, *41*, 748–755.
(22) Kalogeris, E.; Sanakis, Y.; Mamma, D.; Christakopoulos, P.; Kekos, D.; Stamatis, H. *Enzyme Microb. Technol.* **2006**, *39*, 1113–1121.
(23) Gokhale, R. S.; Agarwalla, S.; Francis, V. S.; Santi, D. V.; Balaram, P. *J. Mol. Biol.* **1994**, *235*, 89–94.
(24) Das, M.; Kobayashi, M.; Yamada, Y.; Sreeramulu, S.; Ramakrishnan, C.; Wakatsuki, S.; Kato, R.; Varadarajan, R. *J. Mol. Biol.* **2007**, *372*, 1278–1292.
(25) Bjørk, A.; Dalhus, B.; Mantzilas, D.; Sirevg, R.; Eijsink, V. G. H. *J. Mol. Biol.* **2004**, *341*, 1215–1226.
(26) Andersson, M. M.; Hatti-Kaul, R. *J. Biotechnol.* **1999**, *72*, 21–31.
(27) Bryjak, J. *Bioprocess Eng.* **1995**, *13*, 177–181.
(28) Andersson, M. M.; Breccia, J. D.; Hatti-Kaul, R. *Biotechnol. Appl. Biochem.* **2000**, *32*, 145–153.
(29) Mateo, C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. *Biotechnol. Bioeng.* **2000**, *68*, 98–105.
(30) Fuentes, M.; Pessela, B. C. C.; Maquiese, J. V.; Ortiz, C.; Segura, R. L.; Palomo, J. M.; Abian, O.; Torres, R.; Mateo, C.; Fernandez-Lafuente, R.; Guisan, J. M. *Biotechnol. Prog.* **2004**, *20*, 1134–1139.
(31) Migneault, I.; Dartiguenave, C.; Bertrand, M. J.; Waldron, K. C. *BioTechniques* **2004**, *37*, 790–802.
(32) Betancor, L.; Lopez-Gallego, F.; Hidalgo, A.; Alonso-Morales, N.; Mateo, G.D.-O.C.; Fernandez-Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.* **2006**, *39*, 877–882.
(33) Lopez-Gallego, F.; Betancor, L.; Hidalgo, A.; Alonso, N.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2005**, *37*, 750–756.
(34) Fernandez-Lafuente, R.; Rosell, C. M.; Alvaro, G.; Guisan, J. M. *Enzyme Microb. Technol.* **1992**, *14*, 489–495.
(35) Bolivar, J. M.; Cava, F.; Mateo, C.; Rocha-Martín, J.; Guisdan, J. M.; Berenguer, J.; Fernández-Lafuente, R. *Appl. Microb. Biotechnol.* **2008**, *80*, 49–58.
(36) Filippova, E. V.; Polyakov, K. M.; Tikhonova, T. V.; Stekhanova, T. N.; Boiko, K. M.; Sadykhov, I. G.; Tishkov, V. I.; Popov, V. O.; Labru, N. *Crystallogr. Rep.* **2006**, *51*, 627–631.
(37) Tishkov, V. I.; Popov, V. O. *Biochemistry (Moscow)* **2004**, *69*, 1252–1267.
(38) Zhao, H.; Van der Donk, W. *Curr. Opin. Biotechnol.* **2003**, *14*, 583–539.
(39) Van der Donk, W.; Zhao, H. *Curr. Opin. Biotechnol.* **2003**, *14*, 421–426.
(40) Basu, A. K.; Chattopadhyay, P.; Roychudhuri, P.; Chakraborty, R. *Biosens. Bioelectron.* **2006**, *21*, 1968–1972.
(41) Rodriguez, B. B.; Bolbot, J. A.; Tothill, I. E. *Biosens. Bioelectron.* **2004**, *19*, 1157–1167.

(42) Bolivar, J. M.; Wilson, L.; Ferrarotti, S. A.; Fernandez-Lafuente, R.; Guisan, J. M.; Mateo, C. *Biomacromolecules* **2006**, *7*, 669–673.
(43) Betancor, L.; Lopez-Gallego, F.; Hidalgo, A.; Fuentes, M.; Podrasky, O.; Kuncova, G.; Guisan, J. M.; Fernandez-Lafuente, R. *Biomacromolecules* **2005**, *6*, 1027–1030.
(44) Matto, M.; Husain, Q. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 1316–1323.
(45) Scully, P. J.; Betancor, L.; Bolyo, J.; Dzyadevych, S.; Guisan, J. M.; Fernandez-Lafuente, R.; Jaffrezic-Renault, N.; Kuncovaí, G.; Mateĕec, V.; O'Kennedy, B.; Podrazky, O.; Rose, K.; Sasek, L.; Young, J. S. *Meas. Sci. Technol.* **2007**, *18*, 177–186.
(46) Betancor, L.; Lopez-Gallego, F.; Hidalgo, A.; Alonso-Morales, N.; Fuentes, M.; Fernandez-Lafuente, R.; Guisan, J. M. *J. Biotechnol.* **2004**, *110*, 201–207.
(47) Betancor, L.; Fuentes, M.; Dellamora-Ortiz, G.; Lopez-Gallego, F.; Hidalgo, A.; Alonso-Morales, N.; Mateo, C.; Guisan, J. M.; Fernandez-Lafuente, R. *J. Mol. Catal. B: Enzym.* **2005**, *32*, 97–101.
(48) Mateo, C.; Palomo, J. M.; Fuentes, M.; Betancor, L.; Grazu, V.; Lopez-Gallego, F.; Pessela, B. C. C.; Hidalgo, A.; Fernaíndez-Lorente, G.; Fernandez-Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.* **2006**, *39*, 274–280.
(49) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
(50) Snyder, L.; Sobocinski, P. Z. *Anal. Biochem.* **1975**, *64*, 284–288.
(51) Blanco, R. M.; Guisan, J. M. *Enzyme Microb. Technol.* **1989**, *11*, 360–366.

BM801162E