Structural and Functional Stabilization of L-Asparaginase via Multisubunit Immobilization onto Highly Activated Supports

Victor M. Balcão,[†,‡] Cesar Mateo,[§] R. Fernández-Lafuente,[§] F. Xavier Malcata,[*,†] and José M. Guisán[§]

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal, Universidade Fernando Pessoa, Praça 9 de Abril, P-4249-004 Porto, Portugal, and Instituto de Catálisis y Petroquímica, CSIC, Universidad Autónoma, Campus de Cantoblanco, E-28049 Madrid, Spain

A new protocol for the stabilization of the quaternary structure of multimeric enzymes has been attempted using as model enzyme (tetrameric) L-asparaginase from *Escherichia coli*. Such strategy is based upon multisubunit covalent immobilization of the enzyme onto activated supports (agarose-glutaraldehyde). Supports activated with different densities of reactive groups were used; the higher the density of groups, the higher the stabilization attained. However, because of the complexity of that enzyme, even the use of the highest densities of reactive groups was not enough to encompass all four subunits in the immobilization process. Therefore, a further chemical intersubunit cross-linking with aldehyde-dextran was pursued; these derivatives displayed a fully stabilized multimeric structure. In fact, boiling the modified enzyme derivative in the presence of sodium dodecyl sulfate and β-mercaptoethanol did not lead to release of any enzyme subunit into the medium. Such a derivative, prepared under optimal conditions, retained ca. 40% of the intrinsic activity of the free enzyme and was also functionally stabilized, with thermostabilization enhancements of ca. 3 orders of magnitude when compared with its soluble counterpart. This type of derivative may be appropriate for extracorporeal devices in the clinical treatment of acute leukemia and might thus bring about inherent advantages in that all subunits are covalently bound to the support, with a longer half-life and a virtually nil risk of subunit release into the circulating blood stream.

Introduction

The problem of enzyme stability acquires a special relevance when such complex enzymes as multimeric proteins are employed, because these proteins are composed of several subunits that must be properly assembled to display catalytic activity. Such enzymes are mainly inactivated via subunit dissociation phenomena, which may be accelerated under several experimental conditions (viz., certain pH values, ionic strengths and temperatures) (*1*). The stabilization of the quaternary structure of immobilized multimeric enzymes may thus attract a great interest in many situations, viz., prevention of enzyme inactivation arising from subunit dissociation (*1*). This may indeed be a key feature for using said enzymes in biomedical applications, where the release of enzyme subunits may promote not only enzyme inactivation but also undesired allergenic reactions. Further elimination of such noncovalently bound subunits could be accomplished via washing of the immobilized enzyme derivative under dissociating conditions.

To achieve the stabilization of multimeric enzymes, it is necessary to use an immobilization system specifically designed to address this goal: supports possessing large internal surfaces with a high level of activation (to permit the interaction between the enzyme subunits and the groups in the support) coupled with large immobilization periods (because the correct alignment between the reactive groups in the support and the enzyme may be somewhat complicated) are useful in this particular (*2*, *3*). However, even when a good immobilization system/strategy is used, it may be geometrically impossible to achieve the full stabilization of some multimeric enzymes, e.g., when the enzyme is tetrahedric with one subunit always placed away from the plane defined by the other three subunits. In this case, further chemical cross-linking with multifunctional polymers may permit achievement of full stabilization of the multimeric structure, irrespective of its chemical complexity. The use of polyfunctional macromolecules to achieve such intersubunit cross-linking brings about several advantages: the large size of the polymers may prevent cross-linking/unipoint modification competence; the involvement in the reaction of residues placed in different subunits is likely to occur; and the distance among distinct residues in the protein turns out not to be critical. A deeper discussion on this topic may be found elsewhere (*3*).

In the present research effort, L-asparaginase (EC 3.5.1.1, L-Asnase for short) has been selected as a multimeric model enzyme to test such a strategy of immobilization. This enzyme is a tetrameric protein

* To whom correspondence should be sent.
† Universidade Católica Portuguesa.
‡ Universidade Fernando Pessoa.
§ Universidad Autónoma.

produced commercially by *Acinetobacter glutaminasificans*, *Pseudomonas* spp., *Escherichia coli* and *Erwinia chrysanthemi*, and it hydrolyzes L-asparagine into L-aspartic acid and ammonia, a reaction that under physiological conditions is essentially irreversible (*4*).

Said enzyme is widely used on the clinical level as an antitumoral agent in the treatment of acute leukemia and lymphosarcoma (*4*−*16*). It is effective against neoplasias that require asparagine and obtain it from circulating (plasmatic) pools (*17, 18*), presumably because the cancer cells have diminished expression of asparagine synthetase (*17*). The current intrusive therapy makes use of endovenous administration of said soluble enzyme but is effective only to a certain degree owing to two major shortcomings: L-Asnase circulates in the blood system for a short time before being taken up and broken down; and its presence triggers adverse immunological side-effects because of the presence of an alien protein inside the human body (the severity of which may range from mild allergenic reaction to anaphylactic shock).

The development of strategies that will eventually permit structural and functional stabilization of L-Asnase via immobilization onto highly activated supports may increase its biomedical applicability. Such derivatives could indeed be utilized in extracorporeal devices to eliminate asparagine from the plasma (*19, 20*), and in doing so they would exhibit two clear advantages: the structural stabilization would prevent release of enzyme subunits into the blood and hence would greatly decrease the risks of allergenic reactions; and the functional stabilization would increase the useful life of the extracorporeal device and hence decrease operating costs.

In the present research work, the production of derivatives of L-Asnase from *E. coli* with full stabilization of its multimeric, three-dimensional architecture has been studied via covalent multisubunit immobilization onto highly activated agarose supports, followed by solid-phase intersubunit cross-linking with polyaldehydes obtained from high molecular weight dextran.

Materials and Methods

Materials. ***Enzymes.*** Two enzymes were employed, L-Asnase (type II) from *E. coli*, from Fluka (Steinheim, Germany), which was subject to immobilization, and glutamate dehydrogenase, from bovine liver, from Boëhringer Mannheim (Germany), which was nuclear in the enzymatic assay.

Chemicals. Cross-linked amino agarose beads (4%) were kindly donated by HISPANAGAR S.A. (Burgos, Spain) and prepared as described elsewhere (*2, 28*). Agarose-glutaraldehyde was prepared as described previously (*29*). Glycidol (2,3-epoxy-1-propanol), L-asparagine monohydrate, β-NADH, TRIZMA base, TRIZMA hydrochloride, boric acid, lauryl sulfate (sodium dodecyl sulfate), β-mercaptoethanol, ammonium persulfate, sodium thiosulfate, TEMED (*N′N′N′N*-tetramethylethylenediamine), ethylenediamine, formaldehyde, Schiff's (fuchsin-sulfate) reagent, sodium acetate trihydrate and sodium borohydride were all from Sigma (St. Louis MO). α-Ketoglutarate (disodium salt) was purchased from Boehringer Mannheim. Sodium metaperiodate and silver nitrate were purchased from Merck (Darmstadt, Germany). Prosieve 50 gel solution (modified acrylamide preparation for electrophoresis) was obtained from FMC Bioproducts (Rockland MA). Sodium hydroxide, sodium hydrogen phosphate (monohydrate) and glycerol (87% in water) were all purchased from Panreac Química (Barcelona, Spain). Glutaraldehyde solution (25% in water) and dextran from *Leuconostoc* spp. (20,000 Da) were from Fluka. Aldehyde-dextran was prepared as described by Guisán et al. (*30*). Low molecular weight markers for electrophoresis were purchased from Amersham Pharmacia Biotech (Piscataway NJ). Tap water was purified in a Milli-Q Plus 185 system (Molsheim, France) to a final conductivity of ca. 18.2 MΩ cm^{-1}.

Analytical Equipment. All spectrophotometric readings were carried out using quartz cuvettes in a UVIKON 930 UV−vis spectrophotometer, coupled with a KP91-00580 magnetic stirrer unit for the cuvette (Kontron Instruments, Madrid, Spain).

Experimental Procedures. ***Activity Assay for L-Asnase.*** The activity assay developed for L-Asnase is based on the production of ammonia during hydrolysis of L-asparagine, which is degraded thereafter by glutamate dehydrogenase with concomitant oxidation of β-NADH; disappearance of β-NADH is then monitored spectrophotometrically at 340 nm. The molar extinction coefficient (MEC) of β-NADH was determined to be 4.266 μmol^{-1} cm^{-1}. The improved assay conditions comprise the sequential addition, to a quartz cuvette containing a magnetic rod, of the following reagents: 2 mL of an aqueous solution that is 50 mM in NaH_2PO_4 (pH 7), 1.1 mM in L-asparagine and 0.11 mM in α-ketoglutarate; 25 μL of an aqueous solution that is 50 mM in NaH_2PO_4 (pH 7) and 43.4 mM in β-NADH; and 50 μL of an aqueous solution that is 50 mM in NaH_2PO_4 (pH 7), 50% (v/v) in glycerol and 8.81 μM in glutamate dehydrogenase from bovine liver. The mixture in the cuvette, thermostated at 25 °C, was magnetically stirred for ca. 3 min, and the activity assay was initiated by addition into the cuvette of a 100-μL aliquot of the L-Asnase solution (which was to be tested for activity, typically with an enzyme concentration of 3.86×10^{-8} M); immediately after addition, absorbance at 340 nm was monitored for 10 min. Since the activity of L-Asnase is directly proportional to the rate of ammonia production, and since the large excess of glutamate dehydrogenase degrades it quantitatively with concomitant oxidation of β-NADH, the negative slope of the (decreasing) linear part of the absorbance-vs-time plot provides a measure of the activity of L-Asnase.

Immobilization of L-Asnase onto Agarose-Glutaraldehyde Supports. The preparation of these derivatives encompassed addition of 11.75 mL of aqueous 87% (v/v) glycerol onto a plastic flask, followed by homogenization with 11.75 mL of 25 mM NaH_2PO_4 (pH 7). To the resulting solution was added 220 μL of 4.2 mg L-Asnase/mL glycerol suspension. Following mild homogenization, 5 g of agarose-glutaraldehyde (after activation with either 5 or 40 μmol aldehyde groups/mL gel) was added to this solution, and the flask was stoppered tightly and placed in an orbital shaker at 4 °C. Samples of both the suspension and the supernatant were regularly withdrawn and assayed for activity, together with a blank (which consisted of a sample of enzyme solution withdrawn prior to addition of the support). The immobilization procedure was terminated by providing reduction of the derivative through addition of 29 mL of 100 mM $NaHCO_3$ (pH 10.1) (kept at ca. 4 °C for at least 1 h), adjustment of pH to 10 using concentrated Na_2CO_3 solution, addition of 1 mg of $NaBH_4$ per mL of gel, and mild paddle agitation for ca. 45 min. After this period, the derivative was washed with 500 mL of deionized water.

Modification of Immobilized Enzyme. A given amount of derivative (2 g of L-Asnase/agarose-glutaraldehyde activated with 40 μmol aldehyde groups/mL gel)

was suspended in 5 mL of 200 mM NaH_2PO_4 (pH 6.9) at 4 °C; 15 mL of aldehyde dextran solution, duly oxidized and dialyzed as described elsewhere (*30*), was then added and the resulting suspension was gently homogenized (via an end-over-end rotator) for ca. 14 h. After this period, the modified enzyme derivative was once again reduced: the volume of the suspension was increased 5-fold using 100 mM $NaHCO_3$ (pH 10.1), followed by addition of 1 mg of $NaBH_4$ per mL (to reduce the remaining aldehyde groups, as well as the aldehyde-amine bonds). The suspension was then paddle-agitated for ca. 45 min in an ice bath, after which the derivative was washed with 500 mL of deionized water. The enzyme-dextran bonds were thus transformed into highly stable secondary amine bonds (*24*).

Analysis of the Stabilization of the Quaternary Structure of the Enzyme. To check the stabilization of the quaternary structure of the protein, various enzyme derivatives (using in each case 0.5 g of gel, containing 44−83 μg protein/mL gel) were boiled in one volume (0.5 mL) of 2% (v/v) β-mercaptoethanol and sodium dodecyl sulfate (*2*). This treatment, suggested by Fernández-Lafuente et al. (*3*), releases from the support any enzyme subunit that was not covalently bound to it, while it is unable to break the enzyme-support attachment (or enzyme-dextran bonds, for that matter); all the bonds established between the enzyme and the support (or dextran) are indeed very stable secondary amino bonds and stand still at 110 °C in 6 M NaCl (*3*, *23*, *31*). In this way, any molecule that was not covalently attached to the support (directly or via cross-linking with one subunit already attached covalently to the support) is released into the medium. Then, SDS−PAGE analysis of the supernatant was performed, and the gel was stained with Coomassie Blue (when quantification of the subunits released into the supernatant was sought) and analyzed by densitometry or developed by silver staining (thus allowing detection of as little as 1% of subunits released from the support).

Evaluation of Thermal Stability. The thermal stability of the several L-Asnase derivatives prepared was assessed via preparation of independent suspensions in 50 mM NaH_2PO_4 (pH 7), using in all cases a suitable amount of derivative so as to give the same protein concentration as for the soluble enzyme (i.e., 20.8 μg protein/mL suspension). These suspensions were then assayed for activity and subsequently placed in a water bath at 50 °C. Samples of all suspensions were withdrawn regularly and duly assayed for activity.

Results and Discussion

Activity. A new bi-enzymatic assay that permits the continuous (and rather simple) detection of asparaginase activity was developed and duly assessed. When using an excess of at least 10-fold of glutamate dehydrogenase, the activity detected was directly proportional to the amount of asparaginase (5−24 U) added to the reaction medium in the range studied. Moreover, in the very same range of activities, an increase in the amount of the second enzyme did not promote an increase in the activity observed. Remember that one activity unit (U) is defined here as the number of micromoles of product liberated (or micromoles of substrate hydrolyzed) per minute, at 25 °C and pH 7; the specific activity of the enzymatic preparation (i.e., 100 μL of sample of a 3.86×10^{-8} mol L-asparaginase/L solution) was 223.2 U/mg L-Asnase.

Immobilization of the Enzyme onto Agarose-Glutaraldehyde Supports. The results obtained in the course of immobilization of L-Asnase onto low and highly activated agarose-glutaraldehyde supports are displayed in Figure 1a and 1b, respectively.

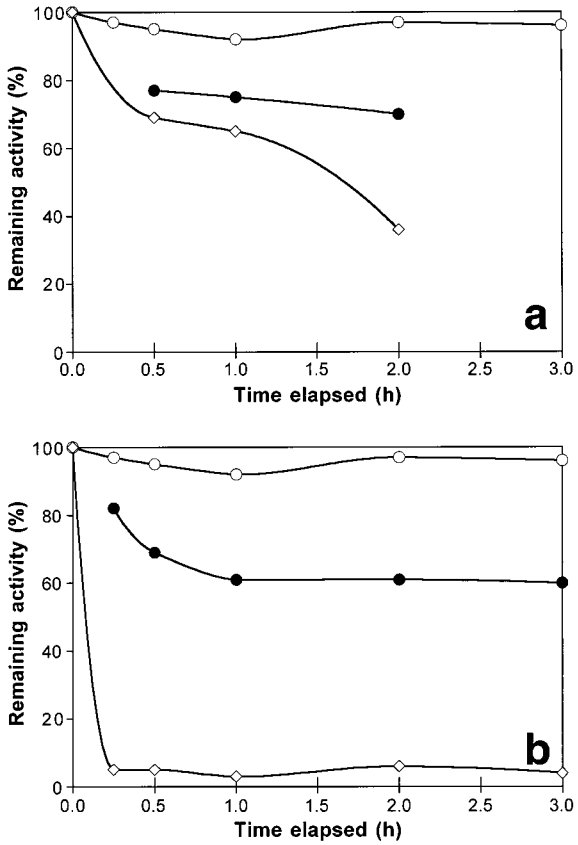

Figure 1. Evolution of L-Asnase activity during immobilization onto agarose-glutaraldehyde supports activated with (a) 5 and (b) 40 μmol aldehyde groups/mL gel. Data points represent the remaining fractional activity of the reference solution (○), the suspension (●), and the supernatant (◇).

The enzyme was very stable under the processing conditions prevailing during immobilization and retained more than 90% of its initial activity by 2 h of incubation. Hence, when using poorly activated supports (with only 5 μmol aldehyde groups/mL gel, see Figure 1a), only a small percentage of the enzyme became immobilized on the support (i.e., ca. 50% of the protein available), and the activity of the enzyme did not seem to be affected by the immobilization procedure.

When using the highly activated support (with 40 μmol aldehyde groups/mL gel, see Figure 1b), the immobilization rate was very high; all enzyme was essentially immobilized after only a few minutes of contact of the support with the enzyme solution. The immobilization on these supports seemed to promote a decrease on the enzyme activity (by ca. 40%), perhaps derived from establishment of some degree of multipoint immobilization, which could promote some distortion on the quaternary structure of the enzyme.

When the immobilization was performed at high ionic strength, the immobilization rate was much lower (only 37% of the enzyme was indeed immobilized by 2 h of contact of the enzyme solution with the support). On the other hand, glutaraldehyde-reduced supports (where covalent attachment was impossible) adsorbed the protein at a greater speed at low ionic strength but were not able to significantly adsorb the protein at high ionic strength. This ionic adsorption of the enzyme could be related with the high density of amino groups beneath

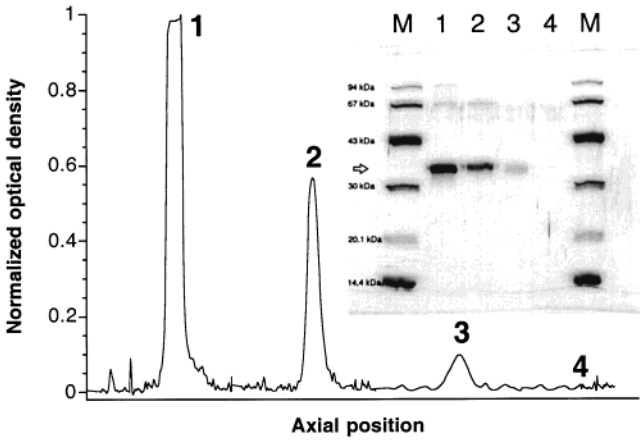

Figure 2. Normalized optical density profile of the SDS−PAGE lanes 1 through 4, in a direction normal to that of the electrophoretic motion (indicated in the inserted picture by a horizontal arrow, ⇒). The inserted picture displays a Coomassie-stained electrophoretogram of soluble L-Asnase and corresponding agarose-glutaraldehyde derivatives, after boiling in the presence of β-mercaptoethanol and sodium dodecyl sulfate (lane M, low molecular weight markers; lane 1, soluble enzyme; lane 2, agarose-glutaraldehyde derivative, obtained with 5 μmol aldehyde groups/mL support; lane 3, agarose-glutaraldehyde derivative, obtained with 40 μmol aldehyde groups/mL support; and lane 4, agarose-glutaraldehyde derivative, obtained with 40 μmol aldehyde groups/mL support and further modified with aldehyde-dextran).

the glutaraldehyde groups (up to 80 μmol/mL support). These results suggest that perhaps the covalent immobilization of this enzyme onto glutaraldehyde supports at pH 7 was slower than its physical adsorption. Therefore, a two-step mechanism of immobilization may be proposed when immobilizing this enzyme on highly activated glutaraldehyde supports at low ionic strength: first, a rapid ionic adsorption of the protein; second, a covalent reaction between protein groups already adsorbed and aldehyde groups at a fairly high rate (due to the proximity of the reactive groups). A similar two-step immobilization strategy is usually followed when immobilizing proteins on epoxy supports (*32*−*36*).

Washing the enzyme derivative at very high ionic strength (e.g., 1 M NaCl) after 2 h of incubation of the enzyme with the support did not release any enzyme from the support (results not shown), thus suggesting that (irreversible) covalent attachment between (at least one subunit of) the enzyme and the support had been fully achieved. In view of the above results, immobilization at low ionic strength was selected as a means to increase the covalent immobilization rate.

A slight decay (by 25−30%) in the activity of the immobilized derivatives (2.535 and 3.195 U/g support for the poorly and highly activated supports, respectively) was noticed after reduction effected by sodium borohydride (*24*, *25*) upon completion of the immobilization procedure, probably as a result of inactivation of (at least) some immobilized enzyme molecules by the alkaline pH employed (viz., 10); at this adverse pH, the soluble enzyme was found to be rather unstable (possibly owing to strong distortions in the three-dimensional architecture ot its globular structure) and loosed ca. 20% of its activity following incubation at 4 °C for 30 min.

Study of the Structural Stabilization of the Quaternary Structure of the Enzyme via Multisubunit Immobilization. Figure 2 depicts the SDS−PAGE analysis of the supernatants, obtained after boiling the free enzyme and the various enzyme derivatives in the presence of β-mercaptoethanol and sodium dodecyl sulfate. The degree of activation of the support exerts a dramatic effect upon the structural stabilization of the enzyme. Using supports prepared with agarose-glutaraldehyde activated with a small concentration of aldehyde groups (viz., 5 μmol aldehyde/mL gel), the derivatives released many enzyme subunits after boiling, as concluded from inspection of lane 2 in Figure 2; using highly activated supports, the intensity of that band decreased. The area of the band in lane 2 (see Figure 2) was calculated as 0.55 OD mm, which represents around 49% of the area of the band in lane 1 (which would account for four subunits of enzyme being released); then, the band in lane 2 would account for between two and three subunits. Derivatives prepared with agarose-glutaraldehyde activated with a high concentration of aldehyde groups, viz., 40 μmol aldehyde/mL gel (see lane 3 in Figure 2) released protein accounting for ca. 23% of the area of the band in lane 1; hence, at most one subunit was actually released from the highly activated derivative (see Figure 2).

Therefore, it seems that the multisubunit attachment of the enzyme to the support was not enough to involve all four subunits, perhaps because of geometrical constraints (e.g., if the tetrameric enzyme is a tetrahedric one, it will be virtually impossible to get the four subunits interacting with a plane surface).

Structural Stabilization of the Quaternary Structure of the Immobilized Enzyme via Intersubunit Cross-Linking. Bearing in mind the previous results, the use of intersubunit cross-linking was attempted with polyfunctional polymers to reach the full stabilization of the immobilized enzyme. This goal was achieved via chemical modification of the immobilized derivatives with aldehyde-dextran molecules, for cross-linking of the free (nonimmobilized) subunit with at least one of the subunits already attached to the support. The chemical modification with aldehyde-dextran had minor effects upon the activity of the derivative (ca. 25% decrease in activity) with a further concomitant slight decrease in the enzyme activity caused by the reduction (ca. 10%). This significantly smaller decrease in the enzyme activity compared with the previous cases could be associated with a certain stabilization of the enzyme against the prevailing reducing conditions (i.e., alkaline pH). In fact, protein was not detected at all in the SDS−PAGE analysis of the supernatant obtained after boiling the modified enzyme derivative in the presence of β-mercaptoethanol and sodium dodecyl sulfate (see lane 4 in Figure 2), even when the SDS−PAGE gel was developed with silver nitrate; this means that substantially less than 1% of the immobilized protein did actually desorb from the support. (Special care was obviously exercised in applying adequate volumes of the boiled supernatants, containing similar amounts of protein, in the wells of the SDS−PAGE gels.)

Functional Stabilization of the Enzyme. The results obtained regarding maintenance of activity of the free and immobilized enzyme when incubated at high temperatures (viz., 50 °C) are displayed in Figure 3a and 3b. Suspensions of all derivatives and a solution of soluble enzyme were prepared by employing suitable amounts of derivative, so as to obtain a total volume of 5 mL for each suspension; this produced equal initial activities for all solutions (i.e., 1 U/mL).

Figure 3a indicates that derivatives obtained using poorly activated supports, with only a maximum of 2 subunits attached to the support, were much more stable than the soluble enzyme, thus suggesting that even the immobilization of two enzyme subunits is enough to

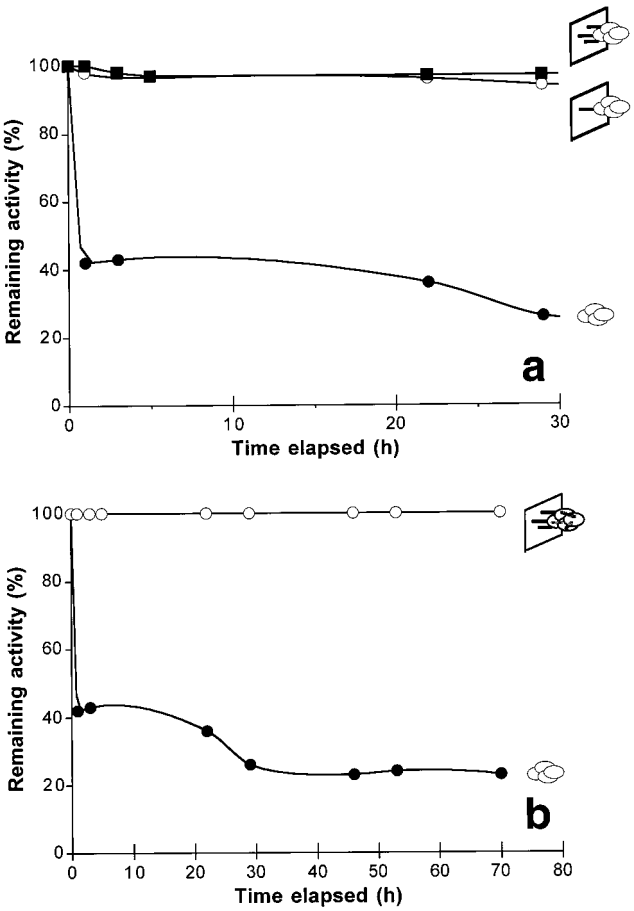

Figure 3. Evolution of remaining fractional activity of soluble and immobilized L-Asnase upon incubation at 50 °C: (a) enzyme in soluble form (●), immobilized onto activated agarose-glutaraldehyde containing 5 μmol aldehyde groups/mL support (○), and immobilized onto activated agarose-glutaraldehyde containing 40 μmol aldehyde groups/mL support (■); and (b) enzyme in soluble form (●), and immobilized onto activated agarose-glutaraldehyde containing 40 μmol aldehyde groups/mL support and further modified with aldehyde-dextran (○).

promote a strong stabilization of the enzyme via rigidification of its backbone and prevention of dissociation effects. The enzyme immobilized onto highly activated supports was quite more stable than the previous (poorly activated) derivatives. The increase in the half-life of the enzyme, achieved via multisubunit immobilization, was higher than 1,000-fold (i.e., the soluble enzyme had a half-life of only 2−3 h at 50 °C, but the derivative maintained its full activity even after 30 h of incubation under similar conditions).

On the other hand, Figure 3b shows that the derivative further cross-linked with aldehyde-dextran presented not only the multimeric structure of the enzyme fully stabilized but also a very high stability at 50 °C. It is hence expected that this derivative should be insensitive to intermittent washing if it were to be used in a continuous reactor. After 80 h of incubation at 50 °C, the derivative further cross-linked with aldehyde-dextran preserved its full activity. Such high stability, together with the structural stabilization achieved, makes this derivative a very good candidate for use in clinical extracorporeal devices.

Conclusions

The covalent reaction involved in immobilization of enzymes onto glutaraldehyde-activated supports may be accelerated by previous adsorption of the protein onto the support by ion exchange. Although the glutaraldehyde groups possess a reactivity sufficient to provide significant immobilization rates, the previous (physical) adsorption of the protein on the support permits increase of the effective concentration of reactive groups, thus contributing to a rapid covalent attachment. Such acceleration of the immobilization may have many advantages, related not only to time keeping but also to prevention of enzyme inactivation by protein−protein interactions (e.g., proteolysis or aggregation), as well as prevention of interaction with inactivating interfaces.

Regarding the model enzyme used in the present study, the methodology proposed, i.e., multisubunit immobilization plus post-immobilization modification with polyfunctional polymers, enabled the stabilization of the quaternary structure of that enzyme. In fact, because of its tetrameric nature, the simple multisubunit immobilization does not necessarily encompass all four subunits simultaneously, so a further intersubunit chemical cross-linking of the enzyme derivative is necessary toward full structural stabilization of the quaternary architecture of the enzyme. The best results point at 40% of the initial intrinsic activity made available to the support, which was fully retained even after 80 h of incubation at 50 °C.

The model reaction chosen for this study, i.e., the enzymatic degradation of L-asparagine under environment-friendly conditions, is a very important reaction for the putative clinical treatment of acute leukemia. Hence, use of these new derivatives in extracorporeal bioreactors would present inherent advantages when compared with the current endovenous administration of the soluble form of enzyme, in that all subunits are covalently bound to the support, with both a longer half-life and a much smaller risk of subunit release into the circulating blood stream.

Acknowledgment

Financial support to V. M. Balcão, via a Post-Doc fellowship by FCT (PRAXIS XXI BPD/16388/98), is hereby gratefully acknowledged. The authors are also grateful to Hispanagar S.A., who kindly donated the activated supports.

References and Notes

(1) Poltorak, O. M.; Chukhary, E. S.; Torshin, I. Y. Dissociative thermal inactivation, stability and activity of oligomeric enzyme. *Biochemistry* (*Moscow*) **1998**, *63*, 360−369.

(2) Guisán, J. M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzyme Microb. Technol*. **1988**, *10*, 375−382.

(3) Fernández-Lafuente, R.; Rodriguez, V.; Mateo, C.; Penzol, G.; Hernandez-Justiz, O.; Irazoqui, G.; Villarino, A.; Ovsejevi, K.; Batista, F.; Guisan, J. M. Stabilization of multimeric enzymes via immobilization and post-immobilization techniques. *J. Mol. Catal. B: Enzym*. **1999**, *7*, 181−189.

(4) Lubkowski, J.; Palm, G. J.; Gilliland, G. L.; Derst, C.; Röhm, K.-H.; Wlodawer, A. Crystal structure and amino acid sequence of *Wolinella succinogenes* L-asparaginase. *Eur. J. Biochem*. **1996**, 241, 201−207.

(5) Oettgen, H. F.; Old, L. J.; Boyse, E. A.; Campbell, H. A.; Philips, F. S.; Clarkson, B. D.; Tallal, L.; Leeper, R. D.; Schwartz, M. K.; Kim, J. H. Inhibition of leukemias in man by L-asparaginase. *Cancer Res*. **1967**, *27*, 2619−2631.

(6) Ohnuma, T.; Holland, J. F.; Freeman, A.; Sinks, L. F. Biochemical and pharmacological studies with asparaginase in man. *Cancer Res*. **1970**, *30*, 2297−2305.

(7) Epp, O.; Steigemann, W.; Formanek, H.; Huber, R. Crystallographic evidence for the tetrameric subunit structure of

L-asparaginase from *Escherichia coli*. *Eur. J. Biochem*. **1971**, *20*, 432−437.
(8) Asselin, B. L.; Lorenson, M. Y.; Whitin, J. C.; Coppola, D. J.; Kende, A. S.; Blakeley, R. L.; Cohen, H. J. Measurement of serum L-asparagine in the presence of L-asparaginase requires the presence of an L-asparaginase inhibitor. *Cancer Res*. **1991**, *51*, 6568−6573.
(9) Story, M. D.; Voehringer, D. W.; Stephens, L. C.; Meyn, R. E. L-asparaginase kills lymphoma cells by apoptosis. *Cancer Chemother. Pharmacol*. **1993**, *32*, 129−133.
(10) Kodera, Y.; Sekine, T.; Yasukohchi, T.; Kiriu, Y.; Hiroto, M.; Matsushima, A.; Inada, Y. Stabilization of L-asparaginase modified with comb-shaped poly(ethylene glycol) derivatives, in vivo and in vitro. *Bioconjugate Chem*. **1994**, *5*, 283−286.
(11) Gaspar, M. M.; Perez-Soler, R.; Cruz, M. E. M. Biological characterization of L-asparaginase liposomal formulations. *Cancer Chemother. Pharmacol*. **1996**, *38*, 373−377.
(12) Jean-François, J.; Fortier, G. Immobilization of L-asparaginase into a biocompatible poly(ethylene glycol)-albumin hydrogel: I: preparation and *in vitro* characterization. *Biotechnol. Appl. Biochem*. **1996**, *23*, 221−226.
(13) Kravtzoff, R.; Desbois, I.; Lamagnere, J. P.; Muh, J. P.; Valat, C.; Chassaigne, M.; Colombat, P.; Ropars, C. Improved pharmacodynamics of L-asparaginase loaded in human red blood cells. *Eur. J. Clin. Pharmacol*. **1996**, *49*, 465−470.
(14) Martins, M. B. A. F.; Gonçalves, A. P. V.; Cruz, M. E. M. Biochemical characterization of an L-asparaginase bioconjugate. *Bioconjugate Chem*. **1996**, *7*, 430−435.
(15) Fernandes, A. I.; Gregoriadis, G. Polysialylated asparaginase: preparation, activity and pharmacokinetics. *Biochim. Biophys. Acta* **1997**, *1341*, 26−34.
(16) Jean-François, J.; D'Urso, E. M.; Fortier, G. Immobilization of L-asparaginase into a biocompatible poly(ethylene glycol)-albumin hydrogel: evaluation of performance in vivo. *Biotechnol. Appl. Biochem*. **1997**, *26*, 203-212.
(17) Swain, A. L.; Jaskolski, M.; Housset, D.; Mohana Rao, J. K.; Wlodawer, A. Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. *Proc. Natl. Acad. Sci. U.S.A*. **1993**, *90*, 1474−1478.
(18) Manna, S.; Sinha, A.; Sadhukhan, R.; Chakrabarty, S. L. Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405. *Curr. Microbiol*. **1995**, *30*, 291−298.
(19) Bernstein, H.; Wheatley, M. A.; Langer, R. S. Extracorporeal reactors containing immobilized species. U. S. Patent 4,-863,611, 1989.
(20) Minim, L. A.; Filho, R. M. Modelling and adaptive control of L-asparaginase reactor. *Comput. Chem. Eng*. **1994**, *18-(suppl.)*, S693−S697.
(21) Penzol, G.; Armisén, P. A.; Fernández-Lafuente, R.; Rodés, L.; Guisán, J. M. Use of dextrans as long and hydrophilic spacer arms to improve the performance of immobilized proteins acting on macromolecules. *Biotechnol. Bioeng*. **1998**, *60*, 518−523.
(22) Fernández-Lafuente, R.; Guisán, J. M. Enzyme and protein engineering via immobilization and post-immobilization techniques. *Rec. Res. Devel. Biotechnol. Bioeng*. **1998**, *1*, 299−309.
(23) Blanco, R. M.; Calvete, J. J.; Guisán, J. M. Immobilization-stabilization of enzymes; variables that control the intensity of the trypsin (amino)-agarose (aldehyde) multipoint attachment. *Enzyme Microb. Technol*. **1989**, *11*, 353−359.
(24) Blanco, R. M.; Guisán, J. M. Stabilization of enzymes by multipoint covalent attachment to agarose-aldehyde gels: borohydride reduction of trypsin-agarose derivatives. *Enzyme Microb. Technol*. **1989**, *11*, 360−366.
(25) Fernández-Lafuente, R.; Rosell, C. M.; Rodriguez, V.; Guisán, J. M. Strategies for enzyme stabilization by intramolecular cross-linking with bifunctional reagents. *Enzyme Microb. Technol*. **1995**, *17*, 517−523.
(26) Guisán, J. M.; Blanco, R. M.; Fernández-Lafuente, R.; Rosell, C. M.; Álvaro, G.; Bastida, A. In *Protein Stability and Stabilization*; van den Tweel, W. J. J., Harder, A., Buitelaar, R. M., Eds.; Elsevier Science: New York, 1993; pp 55−62.
(27) Fernández-Lafuente, R.; Rosell, C. M.; Caanan-Haden, L.; Rodés, L.; Guisán, J. M. Facile synthesis of artificial enzyme nano-environments via solid-phase chemistry of immobilized derivatives: dramatic stabilization of penicillin acylase versus organic solvents. *Enzyme Microb. Technol*. **1999**, *24*, 96−103.
(28) Fernández-Lafuente, R.; Rosell, C. M.; Rodriguez, V.; Santana, M. C.; Soler, G.; Bastida, A.; Guisan, J. M. Preparation of activated supports containing low p*K* amino groups. A new tool for protein immobilization via the carboxyl coupling method. *Enzyme Microb. Technol*., **1993**, *15*, 546−550.
(29) Guisan, J. M.; Penzol, G.; Armisen, P.; Bastida, A.; Blanco, R. M.; Fernández-Lafuente, R.; García-Junceda, E. Immobilization of enzymes acting on macromolecular substrates. Reduction of steric problems. In *Methods in Biotechnology, Vol. 1: Immobilization of Enzymes and Cells*; Bickerstaff, G. F., Ed.; Humana Press: Totowa, NJ, 1997; pp 261−275.
(30) Guisán, J. M.; Rodriguez, V.; Rosell, C. M.; Soler, G.; Bastida, A.; Blanco, R. M.; Fernández-Lafuente, R.; Garcia-Junceda, E. Stabilization of immobilized enzymes by chemical modification with polyfunctional macromolecules. In *Methods in Biotechnology, Vol 1: Immobilization of Enzymes and Cells*; Bickerstaff, G. F., Ed.; Humana Press: Totowa, NJ 1997; pp 289−298.
(31) Bastida, A.; Sabuquillo, P.; Armisen, P.; Fernández-Lafuente, R.; Huguet, J.; Guisán, J. M. A single step purification, immobilization and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. *Biotechnol. Bioeng*. **1998**, *58*, 486−493.
(32) Melander, W.; Corradini, D.; Hoorvath, C. S. Salt-mediated retention of proteins in hydrophobic-interaction chromatography: application of solvophobic theory. *J. Chromatogr*. **1984**, *317*, 67−85.
(33) Smalla, K.; Turkova, J.; Coupek, J.; Herman, P. Influence of salts on the covalent immobilization of proteins to modified copolymers of 2-hydroxyethyl methacrylate with ethylene dimetacrylate. *Biotechnol. Appl. Biochem*. **1988**, *10*, 21−31.
(34) Wheatley, J. B.; Schmidt, D. E. Salt induced immobilization of proteins on a high-performance liquid chromatographic epoxide affinity support. *J. Chromatogr*. **1993**, *644*, 11−16.
(35) Wheatley, J. B.; Schmidt, D. E. Salt induced immobilization of affinity ligands onto epoxide-activated supports. *J. Chromatogr. A* **1999**, *849*, 1−12.
(36) Mateo, C.; Fernández-Lorente, G.; Abian, O.; Fernández-Lafuente, R.; Guisán, J. M. Multifunctional epoxy-supports: a new tool to improve the covalent immobilization of proteins. The promotion of physical adsorptions of proteins on the supports before their covalent linkage. *Biomacromolecules* **2000**, in press.