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Selective oxidation: stabilisation by multipoint attachment of ferredoxin NADP$^+$ reductase, an interesting cofactor recycling enzyme

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Abstract

Ferredoxin–NADP$^+$ reductase (FNR, EC 1.18.1.2) is an enzyme that is able to catalyse the oxidation of NADPH + H$^+$. A strategy to prepare industrial derivatives of this enzyme for use as an 'NADP' regenerating enzyme in oxidizing reactions is presented. The strategy is based on a strictly controlled process of multipoint covalent attachment between the enzyme, via its amino groups, and a pre-existing solid activated with a monolayer of simple aldehyde groups linked by a space-arm of moderate length to the surface of the support. Controlling the variables which may have an influence in the multi-interaction process, we have prepared a number of enzyme derivatives with very different activity/stability properties.

The 'optimum derivative' was found to be much more stable than its corresponding soluble enzyme under all the denaturation conditions assayed (high temperatures, extreme pH, organic solvents, etc.). Because of the excellent properties of this enzyme derivative, we can regenerate NADP$^+$ by using molecular oxygen directly as the oxidizing agent under a wide range of conditions. Coupling this oxidative system to other NADP-dependent redox enzymes, we should be able to develop a very specific and selective oxidative procedure under very mild oxidizing conditions.

Keywords: Cofactor regeneration; Enzyme stabilization ; Enzymic oxidation; Ferredoxin–NADP$^+$ reductase; Immobilization of enzymes; Reductase

1. Introduction

Nowadays, there is a growing interest in the use of enzymes as industrial catalysts [1,2]. This is because enzymes have several advantages when compared with conventional catalysts: they are able to catalyse very selective and specific processes, under very mild conditions. Therefore, enzymes appear to be 'almost-ideal' catalysts for processes involving complex, chiral and labile compounds (e.g, fine chemicals, modification of pharmaceuticals, etc.).

However, industrial implementations of these exciting prospects constitute a complicated problem which require a multidisciplinary approach [3]. In fact, enzymatic processes are very complicated, because of several added complexities: complex substrates, complex process, and complex catalysts (enzymes are labile and soluble macromolecules).

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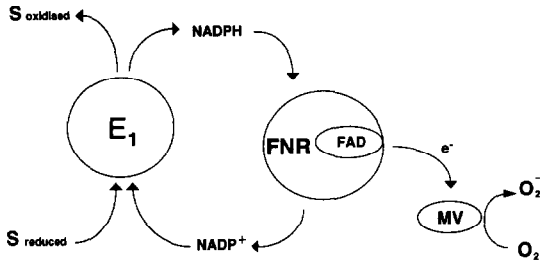

Scheme 1. Oxidation of NADPH mediated by FNR in the presence of methyl viologen.

The use of enzymes in selective enzymic oxidations appears to be even more complicated, because redox enzymes (e.g., hydroxylases, dehydrogenases, etc.) frequently require the use of expensive cofactors (e.g. NAP^+ or $NADP^+$) which should be easily regenerated for an economically feasible process [4]. The use of these cofactors requires the employment of a second enzyme able to continuously recycle the oxidized cofactor, preferably by using an inexpensive sacrificial oxidizing substrate. This 'cofactor regeneration' enzyme should be able to catalyse the recycling of these cofactors under a wide range of conditions. Therefore, it would be necessary to have stable and active industrial derivatives of these enzymes. The great number of published papers on strategies to regenerate cofactors by using simple sacrificial substrates (formate dehydrogenase and glucose dehydrogenase, among others) are proof of the high interest in this subject [5–10].

Ferredoxin–$NADP^+$ reductase (FNR, EC 1.18.1.2) is an interesting redox enzyme. This flavoprotein is present in all photosynthetic eukaryotic cells and cyanobacteria. Its biological role is the transfer of electrons from reduced ferredoxin to $NADP^+$, yielding NADPH during the photosynthesis [11]. This enzyme can be easily isolated in relatively large amounts from cyanobacteria [12]. FNR from *Anabaena* PCC 7119 has recently been cloned and expressed in *E. coli* [13].

FNR from *Anabaena* PCC 7119 can oxidize NADPH using viologens as synthetic electron carriers (Bes et al., unpublished data) in vitro (Scheme 1). Since methyl viologen can be directly oxidized by molecular oxygen [14], this enzymatic process if carefully designed, can be used to reoxidize NADPH from molecular oxygen which is the simplest, cheapest and least harmful oxidizing reagent. In this way FNR could be coupled to a number of redox enzymes (that require $NADP^+$ as cofactor) in order to perform direct oxidations using molecular oxygen as substrate (Scheme 1).

Because of the excellent prospects shown by FNR from *Anabaena* PCC 7119 as an industrial enzyme (it can use molecular oxygen directly), we have tried to prepare an industrial FNR-derivative with good activity and stability properties. The immobilisation of the enzyme by multipoint covalent attachment to pre-existing activated solids was the methodology employed, provided a simultaneous solution for the low stability and the solubility of enzymes. However, multipoint covalent attachment between two complex structures, such as a protein and a support, is very complicated. Because of the moderate geometrical congruence between the enzyme surface and the support it is necessary to obtain, in the area of contact between the protein and the support, a very intensive and non-distortive binding of the enzyme to the support. The critical point was found to be the correct choice of the immobilisation system [15]. The reaction between the amino groups of the protein and a monolayer of glyoxyl groups on the surface of the support with structure type 'void volumes surrender by walls' (e.g., agarose, porous glass, etc.) has allowed obtention of very active and very stable derivatives of a number of enzymes [16–19]. The properties that make it a suitable system to obtain a very intensive but non-distortive support–enzyme reaction have been extensively discussed in previous papers [15–19]. The preparation of any enzyme derivative requires the control of the different variables that can affect the enzyme–support multi-interaction (flexibility of the enzyme structure, reactivity of the reactive groups, superficial density of reactive groups on the surface of the support, etc.) to get the best stability/activity properties. In this paper, we present a study of the immobilization

and stabilization of FNR from *Anabaena* PCC 7119 using this method.

2. Materials and methods

2.1. Materials

Ferredoxin–$NADP^+$ reductase was purified from *Anabaena* PCC 7119 as previously described [20] from cells that were grown autotrophically on nitrate. The concentration of the purified protein was determined from the visible absorption spectra at 458 nm (the extinction coefficient was 9.4 $mM^{-1}\,cm^{-1}$). Cross-linked 10% agarose gels and their glyoxyl derivatives were supplied by Hispanagar S.A. (Burgos, Spain; fax. (+34-47)200328). Glyoxyl agarose CL 10B gels containing different concentrations of glyoxyl groups (from 0 to 210 μmol glyoxyl groups/ml support) were prepared by Hispanagar S.A. as previously described [15]. 2,6-dichlorophenol-indophenol (DCPIP) and NADPH were purchased from Sigma Co. (St Louis, MO). Organic co-solvents and all other reagents were commercially available and of reagent grade.

2.2. Enzymic assays

The diaphorase activity of either FNR derivatives or soluble FNR was assayed by recording the decrease in absorbance at 620 nm (using a thermostated and stirring system) produced by the enzymic bleaching of DCPIP in the presence of NADPH [21]. The reaction mixture contained in a final volume of 2 ml, 50 mM Tris/HCl buffer pH 8, 0.1 mM DCPIP and 0.2 mM NADPH. The reaction was initiated by adding 0.75 μg of soluble or immobilized FNR.

2.3. Preparation of enzyme derivatives

In order to avoid diffusion problems during activity assays, low enzyme loads were used (4 μg enzyme/ml gel). In the standard experiments, 10 ml of glyoxyl agarose gel with different activation degree were added to 90 ml of 50 mM sodium bicarbonate pH 10.05 at 25°C. 4 nmol of FNR in 4 ml of 50 mM Tris/HCl buffer (pH 8.0) were also added to the solution. This 'immobilization suspension' was gently stirred and the pH monitored continuously throughout. A control suspension, identical to the insolubilization suspension but containing inert agarose gels (glyoxyl agarose gel completely reduced with borohydride), was also incubated under the same conditions. Aliquots of the supernatant and whole suspension were withdrawn at intervals and activities assayed as described above. When specified, temperature, gel volumes and buffer nature were altered. After determined contact time periods between the immobilized enzyme and the support, derivatives were reduced with borohydride under conditions previously reported [22].

2.4. Stability assays

Stabilization was defined as the ratio between the half life of the problem derivative and the reference (one-point covalent derivative or soluble enzyme). All the experiments were performed in triplicate.

2.4.1. Irreversible thermal inactivation

Soluble or immobilized FNR was incubated in 0.1 M acetate at pH 5, 0.1 M phosphate pH 7 or 0.1 M borate pH 10, at different temperatures. Periodically, aliquots were withdrawn and the residual diaphorase activity was measured using the standard assay.

2.4.2. Stability in the presence of organic solvents

Solutions of different solvent–acetate buffer were prepared, cooled on ice and adjusted to pH 7. The enzyme (soluble or immobilized) was added and the 'inactivation suspension' was incubated at the desired temperature. When biphasic systems were studied, the inactivation suspension was occasionally stirred (300 rpm).

Table 1
Operational stabilisation of FNR by pure immobilisation

Inactivation conditions				Probable mechanism of inactivation	Stabilisation factor
pH	T (°C)	Stirring (rpm)	Organic solvent		
7	60	0	no	unfolding	1–1.2
5	37	0	no	aggregation	> 2,500
7	25	1000	no	gas/liquid interface	> 10,000
7	25	0	aqueous phase saturated with ethyl acetate	unfolding	0.9–1.1
7	25	300	ethyl acetate/ aqueous phase biphasic system	organic/aqueous interphase	> 100
7	25	0	10% methanol	aggregation	> 100

Inactivation of one-point covalently attached derivatives as compared to the soluble enzyme under different conditions (see Methods).

2.4.3. Stability in the presence of gas–liquid interfaces

Magnetic stirring was carried out in 4 ml solutions of FNR derivatives or soluble enzyme (3 μg enzyme/ml suspension) at pH 7 and 25°C. The system consisted of a 2 cm diameter tube provided with a magnetic bar of 1.8 cm length. The rate of stirring was 1,000 rpm.

3. Results

3.1. Preparation of single-point attachment derivatives

As described elsewhere [15], it is possible to immobilize proteins via a single-point attachment ('one-point covalent attached derivatives') by using supports with a density of reactive groups which is so low that each molecule of protein can only find one glyoxyl group under its projected area. Using this method, the only chemical modification that is introduced in the enzyme is the substitution of a primary amino group by a secondary one. Also, no increase in the enzyme structure rigidity should be expected. Therefore, these one-point covalent attached derivatives frequently have properties such as those of the soluble enzyme ('pure immobilised' enzyme derivatives).

FNR was immobilized on gels having 0.5 glyoxyl residues per 1000 A^2. After one hour of immobilisation reaction, only 5% of the enzyme present was bound to the support. The immobilization yield did not increase when the time was increased to 24 h. By decreasing the pH to 7.0, all immobilised enzyme was found to be rapidly released. The loss of activity observed during this period was the same for the immobilization and blank solutions, that is, only 5–10%. These results suggested that the one-point covalent derivative had been obtained, taking into consideration the reversibility of the individual glyoxyl–amine linkages before reduction [15]. This derivative was used as a control for any further study.

3.2. Effect of pure immobilization on the enzyme stability properties

Pure immobilization is not expected to increase either the enzyme structural rigidity or its intrinsic stability. However, as the enzyme is dispersed on a support surface, deleterious phenomena such as inter-protein interactions (aggregation, proteolysis, etc.) or interaction with interfaces are not possible. This can promote an increase in the 'operational stability' of the enzyme derivative under conditions which previously induced irreversible inactivation. The stability of the one-point attached derivative compared with that shown by the soluble enzyme was determined under a variety of different conditions.

3.2.1. Thermostablility at different pH values

Stabilities of soluble and one-point immobilized enzymes were studied and compared at pH 5 and pH 7 (Table 1). At pH 7, no significant

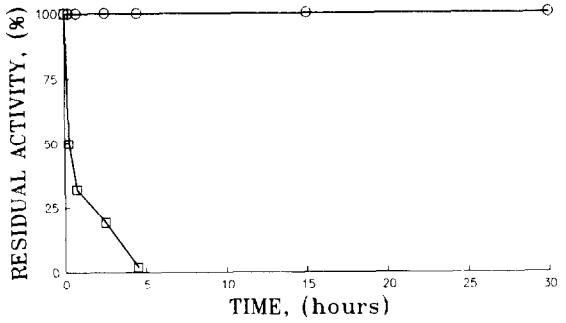

Fig. 1. Stabilisation by 'pure immobilisation' under aggregation conditions. Inactivating conditions were: 37°C, pH 5 and a FNR concentration of 3 μg/ml. □: soluble FNR; ○: one point covalent attachment FNR derivative.

differences in enzyme stability were found. We can assume that under these conditions the unfolding of the enzyme is the main way of enzyme inactivation, and we should not expect an increase of the enzyme 'rigidity' under these processes by 'pure immobilisation'.

The results were notably different when the inactivation was carried out at pH 5 (Fig. 1). At this pH value, soluble enzyme was rapidly inactivated by aggregation, even at moderate temperatures (e.g., all the enzyme activity disappeared at 25°C in only 5 h). However the one-point attached derivative remained fully active after 24 h of incubation at 40°C and at pH 5. This stabilization effect is not due to increased enzyme rigidity, but due to the prevention of enzyme aggregation.

3.2.2. Stability in stirred systems

For this enzyme to be used in industrial oxidizing processes where oxygen is acting as the final electron acceptor, intensive bubbling of air or oxygen into the system may be necessary. Thus the enzyme stability in the presence of gas–liquid interfaces may be a critical point. The stability of FNR to vigorous stirring was thus checked. All enzymic activity was lost after only 30 min of stirring at pH 7 and 25°C. However, the activity of the one-point attached derivative remained invariable after 24 h of incubation under the same conditions.

3.2.3. Stability of the enzyme in organic solvents

Organic co-solvents can promote enzyme inactivation by distorting the protein structure or by promoting its aggregation. Soluble FNR from *Anabaena* PCC 7119 lost 40% of its initial activity after one hour's incubation in an aqueous medium containing 10% methanol. This loss of activity was mainly due to an aggregation process. If the one-point covalent attachment derivative was incubated under the same conditions, it preserved 80% of activity after incubation for 10 h (data not shown). Again, without a real increase in the enzyme rigidity, we have obtained a substantial increase in the operational stability of this enzyme only by pure immobilisation.

Immiscible solvents can be deleterious to enzyme stability because of various effects: On one hand, by direct interaction of solvent molecules dissolved in the aqueous phase which can promote the unfolding or the aggregation of the protein, or on the other, by generating aqueous–organic solvent interfaces. The soluble enzyme showed very different stability when an aqueous phase saturated with organic solvent or a stirred biphasic system was used. When soluble enzyme was incubated in a biphasic system consisting of ethyl acetate/water, it was inactivated in only 15 min, while its incubation in aqueous phase saturated with this solvent promoted the loss of only 40% of the activity under the same conditions. However, the effect on the enzyme activity of the incubation of the one-point covalent attachment derivative in biphasic systems or only in solvent-saturated aqueous phase was the same, and very similar to that found using solvent-saturated aqueous phase and soluble enzyme. Again, although no real stabilisation was detected using this one-point covalent attached derivative, a very significant improvement in the operational stability of the enzyme was found.

According to the results that we have just shown (summarised in Table 1), the enzymic operational stability of FNR (and, probably, of any enzyme) can be greatly increased by preventing intermolecular interactions only by immobilization of the protein inside a porous solid. From a practical

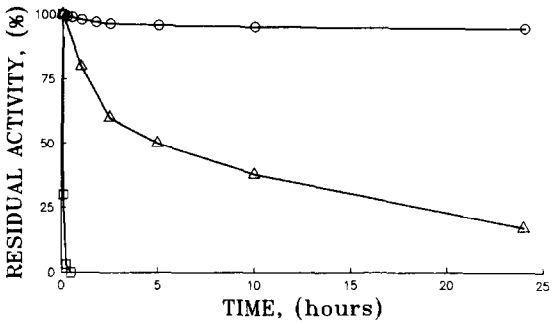

Fig. 2. Immobilisation of FNR on glyoxyl agarose gels. Experiments were carried out as described in Methods. ○: Control suspension; □: supernatant of immobilisation suspension; △: whole immobilisation suspension

point of view, our results strongly suggest the convenience of using immobilized FNR to develop selective oxidations, even when an ultrafiltration reactor was used. The above results also show that the increase in operational stability of the protein may be not related to an increase in the enzyme structural rigidity. Therefore, comparative tests of stability between soluble enzymes and derivatives may be not suitable for the study of multipoint covalent immobilization effects on enzyme stability, because of the possible stabilising effect of the 'pure immobilisation'. For this reason, the one point covalently attached derivative was used as a control.

3.3. Immobilization of FNR from Anabaena PCC 7119 on highly activated supports. Preparation of multipoint covalent attachment derivatives

In order to achieve a high degree of multi-interaction between the enzyme and the support, glyoxyl agarose gels activated with 210 μmol glyoxyl groups per ml of support (glyoxyl surface density around 17 residues per 1000 A^2) were used. The enzyme, under the standard immobilisation conditions, was fully immobilized in only 15 min (Fig. 2). After one hour of the immobilisation reaction, the enzyme was not released after 3 h at pH 7. This fact demonstrated a certain degree of multipoint covalent attachment. Bearing in mind that each individual amine–glyoxyl bond is reversible, the enzyme is immobilised in a kinetically irreversible way only when several bonds have been established between enzyme and support [15]. The activity decreased slowly, but significantly faster than in the blank suspension, during the support–enzyme reaction.

In order to optimize the preparation of an industrial FNR derivative, the effect of different variables which control the stability/activity binomial was checked [23].

3.4. Optimization of the stability/activity binomial

In these studies, stabilities of FNR derivatives were determined at 68°C and pH 7.

3.4.1. Effect of buffer during multipoint covalent attachment

The use of 100 mM borate buffer during the preparation of the enzyme derivative induced less enzyme inactivation compared to incubation in bicarbonate (from 20% to 10% after 1 h of reaction time). This fact was correlated to a lower stabilizing effect promoted by the immobilization process in this buffer: the derivative prepared in bicarbonate buffer was 10-fold more stable than the one prepared in borate (Table 2). These results agreed with those previously found for penicillin G acylase–glyoxyl agarose derivatives [17], and seem to be the consequence of the partial 'blocking' of the aldehyde groups by borate. Further

Table 2
Optimisation of the multipoint covalent attached derivative [a]

Immobilisation conditions			Stabilisation factor	Intrinsic activity (%)
T (°C)	Reaction time (h)	Buffer		
4	1	bicarbonate	10	95–100
25	1	bicarbonate	95–110	85–90
25	1	borate	10	90–95
25	5	bicarbonate	900–1,100	60–65

Stabilisation factors are given as the ratio between the half-lives of multipoint covalent attachment derivatives and one-point covalent attachment derivative. Inactivations were carried out at pH 7 and 68°C.

[a] See Methods.

experiments were carried out using bicarbonate as the reaction buffer.

3.4.2. Effect of temperature

Temperature plays an important role in the multipoint covalent attachment process because it controls the mobility of the enzyme molecule and, therefore, the probability of obtaining correct alignments between the reactive groups of the enzyme and those of the support.

Enzyme derivatives were prepared at 4 and 25°C and their activity–stability binomial studied (Table 2). After one hour of reaction, the derivative prepared at 4°C was only 10-fold more stable than the one-point attached derivative, although the loss of activity was only around 5%. The enzyme derivative prepared at 25°C was 100-fold more stable than the blank derivative. 25°C was chosen as the suitable temperature for the preparation of further derivatives.

3.4.3. Effect of the time of reaction between the support and the enzyme

Support–enzyme multi-interaction time was found to be one of the most important variables that control the degree of multi-interaction [23] because the kinetic limiting step in this process is the correct alignment between the amino residues of the protein molecules and the aldehyde groups on the support. This has been found to be a very slow process. FNR was completely immobilized after 15 min of reaction under the standard conditions (Fig. 2). After this time, it is possible either to reduce the derivatives or leave the already immobilized enzyme to continue the multi-interaction process with the remaining active groups on the support prior to the borohydride reduction of the derivatives. Thus, different derivatives were prepared by varying the time of enzyme–support contact from 15 min to 24 h (Fig. 3). The stability of the enzyme increased from a stabilization factor of 30-fold after 15 min of reaction to 1000-fold after 5 h. An incubation time of 24 h increased the stability up to 2000-fold, but the dramatic loss of activity (85%) makes this procedure unsuitable

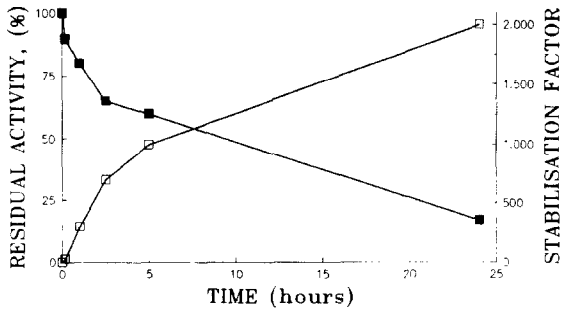

Fig. 3. Effect of the multi-interaction time on the stability/activity properties of immobilised FNR derivatives. CL glyoxyl agarose B (with 210 μmol of glyoxyl groups/ml gel) was used as support. Immobilisation temperature was 25°C, the buffer was 60 mM bicarbonate and the pH 10.05. Inactivations were carried out at pH 7 and 68°C. □: Stabilisation factor, ■: activity.

for the preparation of an industrial catalyst. Thus, the reaction time chosen was 5 h.

In summary, conditions used to prepare stabilized-immobilized FNR derivatives were the incubation of the enzyme in 60 mM bicarbonate buffer pH 10 at 25°C for 5 h. This derivative was around 1,000-fold more stable than the one point covalent attached derivative and preserved 60% of the immobilised activity (Table 2).

3.5. Characterization of FNR-multipoint covalently attached derivatives

3.5.1. Stabilization at different pH values

Optimised FNR derivative were incubated at both acidic and basic pH values (Table 3). At pH 10, the stabilization observed was similar to that found at pH 7 (around 1,000-fold factor, when compared to one-point covalently attached derivatives). At pH 5, the stabilization caused by the multipoint covalent attachment process was significantly higher: the calculated stabilization factor at pH 5 and 63°C was higher than 10,000. This suggested an alternative mechanism of inactivation under different conditions, perhaps involving the desorption of FAD from the enzyme at neutral and basic pH. Further work to clarify the possibly different role of FAD desorption during the FNR inactivation is currently being carried out.

Table 3
Stabilisation exhibited by the 'optimal derivative' under different conditions

Inactivation conditions				Stabilisation factor compared to	
pH	T (°C)	Stirring (rpm)	Organic solvent	Soluble fraction	One-point covalently attached derivative
7	65	no	no	1,000	1,000–1,100
5	65	no	no	>2,000,000	10,000–12,000
10	50	no	no	1,200	1,000–1,100
7	25	1000	no		
7	25	no	aqueous phase saturated with ethyl acetate	900–940	780–850
7	25	300	ethyl acetate/ aqueous phase biphasic system	>1,000,000	800–850
7	25	no	70% ethanol	>2,000,000	6,000–6,500

Inactivation of optimal derivative (see Table 2) as compared with the one-point covalently attached derivatives and the soluble enzyme under different conditions (see Methods).

3.5.2. Stabilisation in the presence of organic solvents

The optimised FNR derivative was very stable in the presence of organic co-solvents. For example in 70% ethanol, the activity of the enzyme derivative remained unaltered after 10 h of incubation. The stabilization factor for the optimum derivative was higher than 6000 (Table 3). Also, the stability in biphasic systems was very high (Table 3).

4. Concluding remarks

There are several features of the FNR immobilisation–stabilisation process upon which we would like to remark.

Firstly, the increase in the enzyme operational stability that immobilisation inside a porous solid promotes by shielding the enzyme from intermolecular processes or interaction with interfaces. This increase may not be due to any effect on the structural rigidity of the enzyme, but this in itself may be very significant. In fact, one-point covalently attached derivatives exhibited the same stability as the soluble enzyme under conditions where the unfolding of the protein was the principle cause in the inactivation process. These enzyme derivatives however did exhibit a very significant operational stabilisation when inactivation was promoted by aggregation or interaction with interfaces. At this point, it is very important point out these effects for these enzymes. Immobilisation of cofactor regenerating enzymes does not appear to be strictly necessary: ultracentrifugation membrane-reactors are frequently used in industrial processes in order to 'retain' modified cofactors (e.g., polyethylene glycol–$NADP^+$), a method which brings about a simultaneous enzyme 'immobilisation'. However, the results presented here have demonstrated the advantages of the use of enzymes immobilised to pre-existing solids even in these reactor types.

By using immobilised enzymes inside a porous support, it is thus possible:

(i) To use vigorous bubbling of oxygen or air to achieve an adequate oxygen concentration, without any loss of activity by protein/gas–liquid interface interaction.

(ii) To use organic media without risk of enzyme precipitation.

(iii) To control the pH of the reaction mixture by titration with concentrated solutions using vigorous stirring, thus avoiding the deleterious effects of pH gradients and gas/liquid interface–protein interaction.

Secondly, the multi-point covalent strategy has increased the rigidity of the enzyme structure. Unlike the results shown for one-point covalent attachment derivatives, stabilising effects have

been found under every inactivation condition tested (high temperatures, drastic pH values, organic solvents, etc.). The stabilisation factor reached values between 1,000 and 10,000-fold, depending on the inactivation conditions employed (Table 3).

Thirdly, the additive affect of stabilisation due to 'pure immobilisation' and by multipoint-covalent attachment should be mentioned. Multipoint covalent attachment derivatives have operational stabilisations which are equal to the stabilising effect of 'pure modification' plus that induced by 'structural rigidification'. In this way, the optimum derivative preserved 60% of initial activity and becomes up to six orders of magnitude more stable than the soluble enzyme under a variety of different conditions (Table 3).

Finally, we point out the excellent possibilities for the use of these enzyme derivatives for cofactor regeneration in many selective oxidative processes: the enzyme has excellent properties and the enzyme derivative presented in this paper exhibits very good stability under a wide range of conditions. Moreover, chemical modification of this enzyme with the viologen *N*-methyl-*N'*-(aminopropyl)-4,4'-bipyridynium has recently been performed in our laboratory (Bes et al., unpublished results). This semisynthetic enzyme was found to be a much more efficient catalyst than the native enzyme, being able to oxidise NADPH with molecular oxygen in the absence of any soluble carrier. Therefore, the use of modified-immobilised-stabilised FNR became a very good alternative for industrial catalysis of oxidizing reactions using molecular oxygen as the sole oxidizing reagent.

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