

Development of lipoic acid activated agarose

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Abstract

A new strategy for the preparation of activated supports containing oxidized lipoic acid (LA) is presented. An amino-agarose gel with low pKa (monoaminoethyl-N-aminoethyl-agarose) was highly substituted with a disulfide ligand (254 μ moles LA/g dried gel) which was subsequently oxidized to disulfide oxides. Oxidation carried out with H₂O₂ gave a support with a large binding capacity for low molecular weight thiols, as demonstrated with glutathione (GSH), achieving 105 μ moles bound GSH per g dried gel. GSH binding was completely reversible under reducing condition. Protein immobilization was assayed with *K. lactis* β -galactosidase. However, although this protein bound readily to the LA-modified support, there was no release of the protein after reduction. We assume that different chemistry was involved in the case of the protein ligand, and this is briefly discussed.

Key words: Lipoic acid, Thioctic acid, Thiol-reactive structures, Disulfide oxides, Thiol immobilization.

Introduction

Lipoic acid (LA) is a widely distributed biomolecule in live organisms and it is known to play a vital role in their metabolism. It has been recognized as an important and powerful biological antioxidant that can directly scavenge free radicals and protect cells from oxidative damage^(1, 2). Oxidative stress is considered to be one of the primary causal factors for ageing and various diseases^(2, 3, 4). Lipoic acid is widely used as a drug for prevention and is administered as a daily supplement for dietary purposes, antiageing, diabetes and cardiovascular disease^(2, 4, 5). LA also functions as a cofactor of oxidative decarboxylation reactions in glucose metabolism to yield energy. To carry out this function, the disulfide group of the lipoic acid dithiolane ring is reduced to its dithiol form, dihydrolipoic acid^(2, 3).

The presence of reactive groups at both ends of the LA molecule makes it potentially useful as a ligand or intermediate for the coupling of many different molecules.

It is reported that LA has a very special chemistry because of its cyclic disulfides. Strain in its five-membered ring structure makes this molecule much more reactive than the similar linear ones^(6, 7). The rate of oxidation of LA is approximately thirty times that of a six-membered cyclic disulfide. Studies on the distribution of lipoic acid in tissues indicates that it is tightly bound to proteins by an amide linkage between its carboxyl group and an ϵ -amino group of a lysine residue in the protein⁽⁸⁾.

The possibility of introducing thiol-reactive structures in the form of disulfide oxides onto solid phases of different types has been extensively studied, and the resulting derivatives display a very high selectivity for the immobilization of thiols^(9, 10).

In this work a different approach was tried for introducing thiol-reactive groups, starting with the immobilization of LA, a ligand carrying a cyclic disulfide. The immobilized ligand was provided with thiol-reactive structures through controlled oxidation of disulfide structures in the lipoic acid. A possible advantage of this approach is that the ligand acts as a spacer arm, which minimises steric hindrance^(11, 12).

Materials

Reduced glutathione (GSH), dithiothreitol (DTT), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 6,8-thioctic acid (lipoic acid), glycidol, ethylenediamine (dihydrochloride), o-nitrophenyl β -D-galactopyranoside (ONPG) and 2,2'-dipyridyl disulfide (2-PDS) were purchased from Sigma (St Louis, MO). Perhydrol (30% hydrogen peroxide) was from Merck (Darmstadt, Germany). β -Galactosidase from *K. lactis* (Maxilact LX 5000) was a gift from Gist Brocades (Cedex, France). PD-10 columns (sephadex G-25) were supplied by Pharmacia BTG-LKB (Uppsala, Sweden). All other chemicals used were reagents of analytical grade.

Methods

Synthesis of MANA-gels

Monoaminoethyl-N-aminoethyl-agarose (MANA-agarose gel) was synthesized according to Fernandez-Lafuente et al⁽¹³⁾.

Lipoic acid (LA) coupling to an amino gel (MANA-gel)

The lipoic acid coupling was carried out essentially as described elsewhere with a molar ratio EDC: LA: NH₂ 3:3:1⁽¹⁴⁾. Lipoic acid solution in acetone (50 mM, 10 ml) was added dropwise with stirring to the same volume of 50 mM EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride) solution in water. The pH was monitored for 1 h and maintained at 4.5. Then, 7.5 g of MANA-agarose gel (containing 375 μ moles NH₂ groups/g dried gel, titrated as reported by Fernandez-Lafuente et al⁽¹³⁾)

was added to the mixture and incubated overnight at room temperature under mild agitation. The gel was sequentially washed with: 1) acetone-water (50:50); 2) water; 3) 1M NaCl, and 4) water. The extent of coupling was determined by elemental S analysis and by thiol titration after reduction with DTT⁽⁹⁾. The complete procedure was repeated once again to obtain a highly substituted amido-LA gel.

Reduction and thiol group analysis

Amido-LA gels were reduced with 100 mM DTT in 0.1M phosphate buffer pH 8.0⁽⁹⁾. The thiol content of the reduced materials was determined spectrophotometrically by titration with saturated 2-PDS solution* as reported by Brocklehurst et al⁽¹⁵⁾ (*1.5 mM 2-PDS solution in 0.1M sodium phosphate buffer, pH 8.0).

Amido-LA gel oxidation with H₂O₂

Oxidation with hydrogen peroxide was carried out by addition of H₂O₂ (30%) (4 aliquots at 0, 30, 90 and 150 min) to the suspended amido-LA gel in 0.2M sodium acetate buffer, pH 5.0. Incubation was continued under shaking to give a total reaction time of 30 hours⁽⁹⁾. Afterwards, titration of thiol-reactive structures was carried out by back titration of glutathione as reported elsewhere⁽⁹⁾.

Glutathione binding

Aliquots of 1.5-2.5 g of drained gels were equilibrated with 0.1M sodium phosphate buffer, pH 7.0, in centrifuge tubes. The total mass in each tube was adjusted to 3.0 g with the same buffer. Aliquots of 3.0 mL of 15 mM glutathione (GSH) dissolved in the same buffer were added to each tube while vortex-mixing. The suspensions were incubated for 30 minutes at 22°C with mixing every 5 minutes. After centrifugation (3000 g, 5 min) 50 µl aliquots of supernatants were mixed with 3.0 mL of 0.25 mM 2-PDS dissolved in 0.1M sodium phosphate buffer pH 8.0 and the absorbances at 343nm were then measured. A blank for the spontaneous oxidation of GSH was run by replacing the gel with an equal volume of phosphate buffer.

The amounts of GSH bound to the oxidized and non oxidized gels were calculated from the difference in absorbance readings at 343nm between GSH blank and the gel supernatants⁽¹⁶⁾. The amount of immobilized glutathione is equivalent to the content of thiol-reactive groups onto the solid phase. More accurate determinations of the bound GSH were performed by total amino acid analysis after thoroughly washing, drying of the gel derivatives, acid hydrolysis and determination of the free amino acids⁽⁹⁾.

*Reduction of *K. lactis* β-galactosidase*

The enzyme was previously reduced with 100 mM dithiothreitol in 0.1M potassium phosphate buffer, pH 8.0 as reported by Ovsejevi et al⁽¹⁷⁾. Afterwards, to get rid of excess of reducing agent, gel filtration (PD-10 columns) was performed in 0.1M sodium phosphate buffer, pH 7.0.

Protein immobilization onto amido-LA oxidized agarose

An aliquot of reduced enzyme (in 0.1M sodium phosphate buffer, pH 7.0) was mixed with the amido-LA-Ox gel and incubated overnight at room temperature under end over end rotation. The protein load was 161 mg of reduced and gel filtered β-galactosidase (*K. lactis*) per g of dried gel. After incubation, the gel was thoroughly washed with: 1) phosphate buffer; 2) 1M NaCl and 3) water. Total amino acid analysis was performed for each gel after drying over P₂O₅.

Reversibility of thiol binding

An aliquot of each gel derivative prepared above (with low and high molecular weight thiols) was treated with 100 mM DTT in 0.1M phosphate buffer, pH 8.0 and washed afterwards using the same protocol as above. Total amino acid analysis was performed for each reduced gel after drying over P₂O₅.

*β-Galactosidase (*K. lactis*) enzymatic assay*

Enzyme activity in solution and onto solid phase was assayed as reported by Ovsejevi et al⁽¹⁷⁾.

The activity of the enzyme solution was assayed at room temperature using 14mM ONPG (o-nitrophenil β-D-galactopyranoside) as substrate in 20 mM potassium phosphate buffer, pH 7.0, containing 2.0 mM MgCl₂ and 0.1M KCl (Activity buffer) and the released o-nitrophenol was determined at 405nm. The immobilized enzyme activity was assayed by incubating 100 µl aliquots of gel suspensions (containing 10 mg of suction-dried gel derivatives) with 3 mL of 28 mM ONPG in activity buffer, using a 1 cm path length cuvette provided with magnetic stirring. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of ONPG per min at 25°C and pH 7.0.

Protein determination

The protein content of the enzyme solution was assayed by measurement of absorbance at 280nm.

Results and discussion

Immobilization of lipoic acid

The coupling of lipoic acid to an amino-agarose gel by the carbodiimide procedure was studied. The method involves the reaction of the carboxyl group in the LA molecule with amino structures in the gel, so that LA is covalently bound by an amide bond to the support (Figure 1).

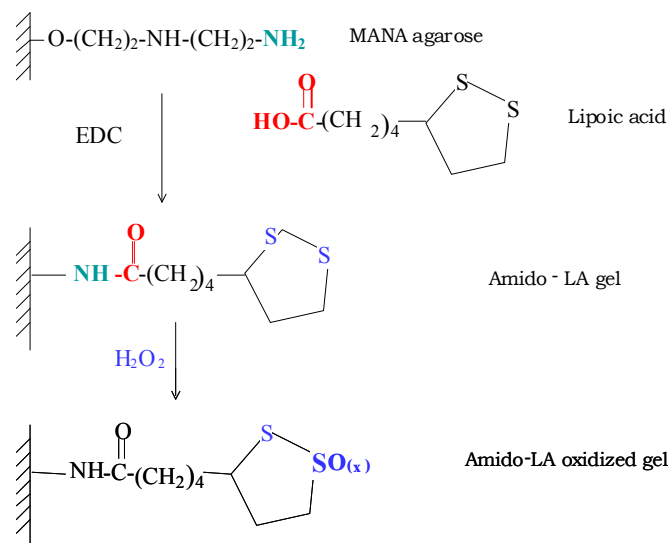


Figure 1.-Lipoic acid coupling to an NH_2 -gel and subsequent oxidation.

The amino-gel chosen was an agarose gel containing monoaminoethyl-N-aminoethyl structures (MANA-gel) which contains primary amino groups with low pKa (6.8)⁽¹³⁾. As it has been well established, the mild conditions of the carbodiimide coupling procedure, requires the presence of very high concentrations of nucleophilic groups in the reaction. Most of the known aminated supports, e.g. aminohexyl AH-Sepharose which have been tried without success, contain a low concentration of aliphatic amino groups with very high pKa values. MANA-gels can be obtained with a high degree of substitution on agarose, and the primary amino group has a surprisingly low pKa value because it is very close to a secondary amino bond. These facts make these gels suitable for carbodiimide activation in mild conditions⁽¹³⁾. The MANA-agarose gel yielded a highly substituted support, with the immobilization of 254 μmoles of lipoic acid per gram of dried gel (Table 1).

The efficiency of the LA coupling was evaluated in two ways, by elemental S analysis of the amido-LA-gel, and by reductive treatment of the same gel with DTT followed by SH titration (Table 1). The discrepancy in the figures obtained with the two analytical methods is most probably due to differences in the gel swelling factors and to exchange reactions occurring simultaneously during the titration procedure. Therefore S analysis was chosen as the more reliable of the two.

Table 1 : Lipoic acid coupling to MANA- agarose gels.

Derivative	$\mu\text{moles LA/g dried gel}$	
	by SH titration	by elemental S analysis
Amido-LA gel (1 st treatment)	182	131
Amido-LA gel (2 nd treatment)	347	254
MANA gel (control) ^(a)	----	0

^(a) MANA agarose gel had 375 $\mu\text{moles NH}_2$ groups/g dried gel.
The results are mean values from duplicate experiments.

Since LA is insoluble in water and the carbodiimide employed (EDC) is insoluble in organic solvents, the coupling was done in a system of acetone: water 1:1, but with low concentrations of each reagent. The procedure was carried out in two consecutive steps, first only 35% of the NH_2 groups in the MANA-agarose gel reacted and, after the second treatment under the same conditions, the gel was highly substituted with ligand (68% of the NH_2 groups were derivatized with LA, calculating percentages

on the basis of elemental analysis). The unmodified amino gel (control), showed almost no S content (Table 1). The coupling technique produced a support with a reasonably high substitution with a disulfide carrying ligand bound through a spacer arm.

Oxidation of immobilized lipoic acid and coupling of glutathione

The chemistry of lipoic acid has been extensively studied, and it is reported that mild oxidation of LA with hydroperoxides converts it to a sulfoxide⁽⁶⁾. The possibility of introducing reactive structures like disulfide oxides (thiolsulfinate or thiolsulfonate groups) onto solid phases by taking advantage of the characteristics of lipoic acid, provides an interesting potential use of thiol coupling. The activation reaction of the immobilized LA was carried out with H₂O₂.

It is reported elsewhere that thiol-reactive structures generated after hydrogen peroxide treatment of thiol- agarose are mainly of thiolsulfonate type⁽⁹⁾. But this might not necessarily be the case with lipoic acid, since the presence of thiolsulfonates has to be considered as equally probable on account of previous reports⁽⁶⁾. The reactivity of the gel cannot be explained by the disulfide groups since the control gel with lipoic acid in its ring form, subjected to the same conditions, showed no reaction.

The oxidation was performed essentially as described for thiol-agarose⁽⁹⁾, and the reaction of the activated gel with GSH (a low molecular weight thiol) was studied. Table 2 shows the number of thiol-reactive structures on the gel oxidized with hydrogen peroxide (amido-LA-Ox gel), represented by the number of μ moles GSH bound/ g dried gel. This last figure was calculated from the difference in absorbance reading at 343 between the GSH blank and the gel supernatants. The stoichiometry of the reaction is the same for any monothiol molecule, each thiol reacts with one disulfide oxide group. Amino acid analysis was also performed and confirmed the results obtained before.

Table 2.- Glutathione immobilization onto agarose-modified gels

Derivative	μ moles GSH bound /g dried gel	
	by titration	by total amino acid analysis of the GSH derivative
MANA-LA gel (control 1)	0	0
Amido-LA-gel ^(a) (control 2)	0	5.8
Amido-LA-Ox-gel ^(b)	149	105.0
Amido-LA-Ox-gel + DTT	----	0

^(a)Starting material, amido-LA agarose had 254 μ moles LA/g dried gel,

^(b)Oxidation was carried out with H₂O₂.

The results are mean values from duplicate experiments

The original amino-gel and the amido-LA-gel without oxidation were used as controls to confirm that the reactivity was due to the oxidized structures. In addition the bound glutathione could be eluted with an excess of DTT. The complete absence of glutathione in the gel after this treatment (confirmed by amino acid analysis) shows that GSH binding was completely reversible. This strongly indicates the disulfide bond nature of the linkages (Table 2).

Protein immobilization onto amido-LA oxidized gels

Protein immobilization onto amido-LA oxidized gels was examined with β -galactosidase from *K. lactis*. A large quantity of protein was applied to the amido-LA oxidized gel, and the derivative obtained had high amounts of immobilized protein (62 mg of protein per g dried gel) bound onto the support, preserving an activity of 956 EU per g dried gel (Table 3).

Table 3.- Immobilization of *K. lactis* β -Galactosidase onto amido-LA-Ox agarose.

Derivative	Immobilized protein (mg/g dried gel)	Immobilization yield (%) [*]	Expressed activity (EU/g dried gel)
MANA-agarose (control 1)	0	0	0
Amido-LA-Ox gel ^(a)	62	48	956

^{*}Determined by difference between applied activity and that remaining in solution.

^(a) Starting material, amido-LA gel with 191 μ moles LA/g dried gel (only first treatment with carbodiimide).

But the most surprising fact was that control gels containing disulfide groups (control 2) also bound these proteins to the same extent. Furthermore, all the protein remained on the solid phase after DTT, NaCl and ethylene glycol treatment, showing a completely irreversible binding, in contrast with the fully reversible binding of GSH. This difference between the behavior of low and high molecular weight thiols must have a reasonable explanation. Perhaps, due to the long spacer arm of the lipoic acid derivative, with many C atoms and some charged groups in the amino gel, there might be a mixture of hydrophobic, ionic and covalent interactions that makes the release of very difficult. If the protein is bound by multiple interactions, when one type of interaction becomes weaker the others might become stronger. The possibility for such interactions is much lower for low molecular weight thiols because of their small size.

Conclusions

Coupling of lipoic acid to an amino gel with low pKa (MANA- agarose gel) gave a support provided with a large number of ligand molecules carrying disulfide groups (254 μ moles of lipoic acid per gram of dried gel). A second step, oxidation of amido-LA gel with hydrogen peroxide, gave a support with large capacity to bind thiols, as demonstrated with GSH (105 μ moles bound per g dried gel), which should thus be useful for the reversible immobilization of low molecular weight thiols. The nature of the reactive groups was not confirmed, but it is reasonable to propose that they are disulfide oxides, since the unoxidized control gel did not show any reaction. Furthermore, release of bound GSH was complete and no GSH was found on the derivative after DTT treatment. Thus, the binding of low molecular weight thiols to the amido-LA oxidized gel was totally reversible. Since previous washings with buffers with different ionic strength and pH failed to remove the bound thiol, we can assume that the bond is of a covalent nature.

β -Galactosidase immobilization onto oxidized amido-LA oxidized gel resulted in a derivative with high amounts of bound protein and high activity (62 mg protein per g of dried gel and 956 EU per g of dried gel), however the coupling was irreversible since DTT, NaCl and ethylenglycol failed to remove the protein from the gel. This may be explained by multipoint interactions of different types, including ionic, hydrophobic and non specific interactions. The sum of these interactions could make the protein binding completely irreversible since when one type of interaction becomes weaker the others might become stronger.

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