Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 449-457

IMMOBILIZATION OF ENZYMES AND AFFINITY LIGANDS TO VARIOUS HYDROXYL GROUP CARRYING SUPPORTS USING HIGHLY REACTIVE SULFONYL CHLORIDES

Kurt Nilsson and Klaus Mosbach

Department of Pure and Applied Biochemistry, Chemical Centre, P.O.B. 740, S-220 07 Lund, Sweden.

Received July 28,1981

SUMMARY

A facile method for the activation of hydroxyl group carrying supports such as agarose, cellulose, diol-silica, glycophase-glass or hydroxyethyl methacrylate gels with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) is described. On reaction of the resulting tresylate containing supports with different enzymes or affinity ligands at neutral pH, overnight and at 4 °C, coupling yields and retained specific activities of up to 80 and 50 %, respectively, were obtained. The bound ligands showed excellent affinity properties. Thus, N^6-(6-aminohexyl)-AMP coupled to diol-silica completely separated a mixture of albumin, lactate dehydrogenase and alcohol dehydrogenase in less than 15 min when the technique of high performance liquid affinity chromatography was employed.

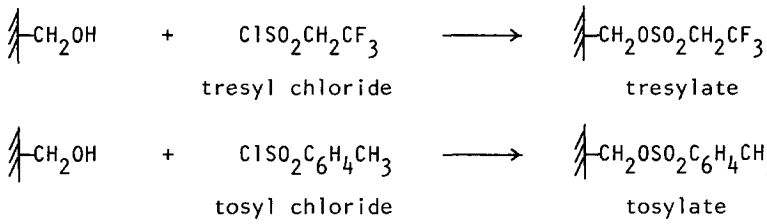
INTRODUCTION

Recently a new method of activating hydroxyl group carrying supports using p-toluenesulfonyl chloride allowing subsequent immobilization of proteins and affinity ligands was described (1-3). This method was shown to have advantages over presently used ones since ligands are bound directly to the carbon atoms of the support and no side reactions occur during activation and coupling. In the literature a number of derivatives of sulfonic esters have been reported to be more reactive than these tosylates including p-nitrobenzenesulfonate, trifluoromethanesulfonate and 2,2,2-trifluoroethanesulfonate (tresylate) (4). In this report we wish to describe an immobilization method based on the latter compound as it allows efficient coupling under very mild conditions and to a number of different supports including those used for high performance liquid chromatography. The reactions involved in immobilization of biomolecules using

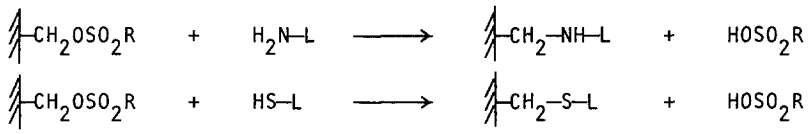
0006-291X/81/170449-09$01.00/0

tresyl chloride or tosyl chloride are believed to be as given in the scheme below:

Activation:



Coupling:



(R = CH_2CF_3 or $C_6H_4CH_3$; L = ligand or enzyme)

MATERIALS AND METHODS

Activation with tresyl chloride was performed at room temperature, principally as described for activation with tosyl chloride (1,2). Sepharose (4B, CL-4B or CL-6B, Pharmacia) was transferred to dry acetone via the following washing steps on a glassfilter funnel: 10 gel volumes of dist. water, 30:70, 60:40 and 80:20 of acetone p.a.:water (V/V), 2x10 volumes of acetone and finally 3x5 volumes dried acetone (dried over molecular sieve, 100 g/3 l acetone). The gel (32 g) was then transferred to a beaker containing 0.1 gel volume dry acetone and pyridine (2 ml/ml tresyl chloride). Under vigorous magnetic stirring 0.2-1.0 ml tresyl chloride (>97 %, Fluka) was added dropwise for 1 min to the gel-suspension. The reaction was continued for 10 min. The gel was then washed with 2x10 gel volumes acetone, 10 volumes of 30:70, 50:50, 70:30 and 85:15 1 mM HCl:acetone and finally with 2x10 volumes of 1 mM HCl and stored at 4 °C until used. The entire activation procedure can be completed within 1.5 h.
Cellulose (Sigmacell, type 100) was activated by the same procedure after swelling in 1 M NaOH for 1 h. To facilitate stirring during activation 0.5 gel volumes of acetone was added.
Diol-silica (1,2-dihydroxy-3-propoxypropylsilyl-silica), prepared from LiChrospher Si 500 (Merck) as described previously (5), LiChrosorb^R Diol (5 µm, Merck) and glycophase-glass (Glycophase-G^{TM}/CPG-40, 5-10 µm, Corning) were initially washed with 90:10 acetone:water (V/V), then washed with dried acetone and treated with tresyl chloride as above, except for using 12 ml of dry acetone/g dry gel to facilitate stirring. After washing of the gels as described above they were ready for use. Alternatively, they could be washed with acetone and sucked dry for storage.
Hydroxyethyl methacrylate (Spheron^R P 100,000, Koch-Light) was treated as the silica gels, however 1 gel volume acetone was added to the settled gel to allow proper stirring during activation.

Coupling. *Trypsin* (bovine pancreas, type III, Sigma), trypsin inhibitor (soybean, type I-S, Sigma), albumin (bovine, fraction V, Sigma) and N^6-(6-aminohexyl)-AMP

(Sigma) were dissolved in cold coupling buffer (either 0.2 M sodium bicarbonate or 0.2 M sodium phosphate, + 0.5 M sodium chloride). One volume of settled activated gel was briefly washed with the cold coupling buffer and added to one volume of ligand solution. Coupling proceeded at 4 °C with gentle agitation. Coupling was terminated by washing with 3x10 gel volumes of coupling buffer, 0.2 M sodium acetate, pH 3, containing 0.5 M sodium chloride, 0.5 M sodium chloride, dist. water and coupling buffer. Hexokinase (yeast, type III, Sigma) was coupled in 0.2 M Hepes buffer, pH 7.0 containing 15 mM $MgCl_2$, and washed as above omitting the acetate buffer. Concanavalin A solution (14.5 mg in 0.5 ml saturated sodium chloride, Miles), was added at 4 °C to 1.1 g wet tresyl Sepharose 4B, which had been rapidly washed with buffer (0.2 M sodium phosphate + 0.5 M sodium chloride, pH 7.5). The gel was washed as recommended for CNBr coupled Concanavalin A (6).
Protein content was determined by amino acid analysis and bound AMP-analogue was determined by uv-measurements (1). Determination of free and immobilized enzyme activities was done spectrophotometrically as described previously (1). The assay system for hexokinase was as given by the Worthington Manual (7).

Activation with CNBr of Sepharose CL-4B was done according to March et al (8). Cyanate groups were determined by the method described by Kohn and Wilchek (9). Coupling of trypsin inhibitor (10 mg/g gel) and [^{125}I] albumin (5 mg/g gel) to to CNBr and tresyl chloride activated Sepharose CL-4B were done at pH 7.5 and 4 °C as described above. Coupling yield and leakage of albumin were determined by measuring [^{125}I] activity of the gel and the collected washings.

Affinity experiments were done with the gels packed in Whatman columns of 0.3 cm diameter. The flow rate was controlled with a peristaltic pump. The absorbance of the eluate was recorded with a Uvicord (Pharmacia). The experiments were done at 4 °C except for the purification of peroxidase which was done at room temperature. Gels used for affinity chromatography were prepared by coupling at pH 7.5 and 4 °C.
Soybean trypsin inhibitor (15 mg) was coupled to 1.5 g Sepharose 4B (0.8 mmol tresyl groups/g dry weight) as described under Coupling. To remove any interfering tresyl groups, the coupled gel was washed with cold 0.1 M Tris-HCl, pH 7.5, for 30 min on a glass filter. A mixture of 4 mg albumin (bovine,type V,Sigma) 2 mg trypsin (bovine pancreas, type III, Sigma) and 2 mg α-chymotrypsin (bovine pancreas, type 1-S, Sigma) in 0.5 ml effluent (0.2 M sodium phosphate, pH 7.2) was applied to 1 g gel containing 7.5 mg inhibitor. The flow rate was 20 ml/h. Peroxidase (horse-radish, grade II, Boehringer) was applied to 0.5 g Sepharose 4B containing 6.5 mg Concanavalin A. The effluent contained 0.1 M sodium acetate pH 6.0, 1 M sodium chloride and 1 mM $MgCl_2$, $MnCl_2$, and $CaCl_2$ each. After application of 0.25 ml enzyme solution (containing 1.25 mg protein the flow was stopped for 30 min to allow equilibration. Fractions were then collected at a flow rate of 10 ml/h.

Preparation of AMP-silica used for High Performance Liquid Affinity Chromatography (HPLAC). LiChrosorb^R Diol (5 µm, Merck), 2.5 g dry weight, washed with acetone as described under Activation with tresyl chloride, was suspended in acetone (10 ml) and pyridine (1 ml) at room temperature. Tresyl chloride (0.5 ml) was added dropwise (1 min) with stirring. The gel was washed after 20 min as described under Activation. N^6-(6-aminohexyl)-AMP (40 mg) was dissolved in 2.5 ml 0.7 M sodium bicarbonate and added to the wet tresyl silica (7 g). Coupling proceeded for 20 h at 20 °C at pH 7.5. After washing with coupling buffer the gel was treated with 0.1 M mercaptoethanol, pH 7.5, for 4 h at 20 °C (treatment with 0.1 M Tris-HCl buffer, pH 7.5, is believed to be a good alternative as was found for Sepharose bound affinity ligands). After washing of the gel as described under Coupling it was washed with acetone, sucked dry and analyzed for nitrogen. It was found that 13.2 mg AMP-analogue/g dry gel had coupled (i.e. 79 % yield).

Table 1. Activation of Sepharose with tresyl chloride.

Type of Sepharose	Amount of tresyl chloride used[a] mmol/g wet gel	Amount of tresyl groups formed[b] mmol/g dry weight
4B	0.15	0.80
4B	0.30	1.28
CL-4B	0.15	0.79
CL-4B	0.30	1.35
CL-6B	0.10	0.45
CL-6B	0.20	0.98
CL-6B	0.30	1.10

a) The reactions were done in acetone for 10 minutes as described in Materials and Methods.
b) Calculated from the sulphur content as determined by elemental analysis.

RESULTS AND DISCUSSION

Activation. The degree of substitution of various Sepharose preparations as a function of different amounts of tresyl chloride used during activation was studied (Table 1). As seen the same substitution pattern was obtained for the different Sepharose preparations. Noteworthy is the high amount of tresyl groups obtained after only 10 min of reaction (>1 mmol/g dry product). The swelling properties of Sepharose did not change during the activation since the settled volume of the gel was unchanged when transferred back to water.

The ratio of fluorine to sulphur, as determined by elemental analysis, corresponded well with the expected numbers. No nitrogen was introduced indicating that no pyridine was incorporated during the reaction. The amount of both S and F decreased by only a few per cent on storage at 4 ^{o}C overnight in 0.1 M phosphate, pH 7.5. This rather high stability contrasts with CNBr activated agarose where the half-life of cyanate ester groups in the aqueous phase at neutral pH is reported to be about 30 min (9). For storage it is recommended to keep the gels in 1 mM HCl at 4 ^{o}C as it has been found that the coupling capacity for enzymes did not decrease after storage for at least three months under these conditions. Alternatively, the gels can be lyophilized or kept in acetone.

Table 2. Coupling of enzymes and affinity ligands to different supports activated with tresyl chloride.

Support[a]	Ligand[d]	Bound ligand mg/g dry support	Yield %	Specific activity relative to soluble enzyme %
Sepharose 4B	Concanavalin A	360	97	
Sepharose 4B	Soybean trypsin inhibitor	195	68	
Sepharose CL-6B	Hexokinase	94	53	26
Sepharose CL-6B	Trypsin	124	70	33[e]
Cellulose	"	61	87	56
Diol-silica[b]	"	24	80	63
Sepharose CL-6B	N^6-(6-amino-hexyl)-AMP	87	32	
Cellulose	"	49	44	
Diol-silica[b]	"	18	36	
Glycophase-glass[b]	"	15	45	
Hydroxyethyl methacrylate[c]	"	10	12[c]	

a) Sepharose 4B and CL-6B and cellulose contained 1.28, 1.1 and 0.5 mmol tresyl groups/g dry weight, respectively. Activation of all polymers lasted for 10 minutes and 1.0 ml pyridine and 0.5 ml tresyl chloride were added to 17 g gel-suspension (i.e. support suspended in acetone, see Materials and Methods).
b) 1,2-dihydroxy-3-propoxypropylsilyl-silica and Glycophase-G™/CPG-40 were used.
c) Spheron P 100,000 (this preparation has a low surface area, which may explain the relatively low coupling yield).
d) Amount of ligand applied/g wet gel during coupling: 13 mg Concanavalin A; 10 mg soybean trypsin inhibitor; 10 mg trypsin; 10 mg hexokinase and 16 mg N^6-(6-aminohexyl)-AMP. Concanavalin A and trypsin inhibitor were coupled for 15 h at 4 °C and at pH 7.5 and hexokinase at pH 7.0. The comparative studies involving trypsin and the AMP-analogue were carried out with the preparations coupled at pH 8.2. The affinity experiments described in the text however were made on gels coupled at pH 7.5.
e) On coupling for shorter time (4 h) 55 % was bound but the specific activity was higher (50 %).

Different hydroxyl group carrying supports were conveniently activated with tresy chloride followed by efficient coupling of enzymes or affinity ligands under mild conditions (Table 2).

Coupling of enzymes. The coupling of trypsin inhibitor under various conditions was studied. As seen from Table 3, high coupling yields (72 %) were obtained at relatively neutral conditions (pH 7.5). For hexokinase pH 7.0 was shown to be optimal whereas trypsin bound more efficiently at a somewhat higher pH (8.0, see also Table 2). The stability of the labile enzyme hexokinase was found to be very

Table 3. Coupling of soybean trypsin inhibitor to tresyl Sepharose 4B (1.28 mmol tresyl groups/g dry weight). Coupling was done at 4 °C.

Amount inhibitor added mg/g wet gel	Time for coupling h	pH	Bound inhibitor mg/g dry support	Yield %
10	15	6.5	70	25
10	15	7.5	195	68
10(20)	15	8.5	211(366)	74(64)
10	15	9.5	223	78
10	15	10.5	195	68
10	1.5	8.5	200	70

high when immobilized to tresyl agarose as no decrease in activity was detected after storage for one month at pH 7.0 and 4 °C, while such storage for the free enzyme in solution resulted in loss of all enzymic activity after a few days. The normal reaction in the immobilization of biomolecules to tresyl group carrying supports is believed to involve nucleophilic displacement at the carbon atom of the matrix. At alkaline conditions (pH > 8.5) it might be possible that substitution of the fluorine of the terminal CF_3 group with the biomolecule in question occurs as a side-reaction. Although immobilization of enzymes or affinity ligands carried out at these high pH values did not adversely affect their performance, to obtain a clearcut nucleophilic displacement, immobilization at pH-values 7-8 is recommended, which anyhow is the pH range of choice, notably for enzymes.

Affinity chromatography. The affinity properties of immobilized trypsin inhibitor were tested by applying a mixture of albumin, α-chymotrypsin and trypsin. On application at pH 7, albumin and inactive molecules of trypsin and α-chymotrypsin appeared in the void volume as one sharp peak. The bound active forms of α-chymotrypsin and trypsin could subsequently be completely eluted by changing to 0.2 M acetate of pH 4.8 and 3.0, respectively. The capacity of a preparation containing 11 mg inhibitor/g wet polymer was shown to be 9 mg trypsin per g of sorbent. The specific binding of the glycoprotein peroxidase (from horse-radish) to Conca-

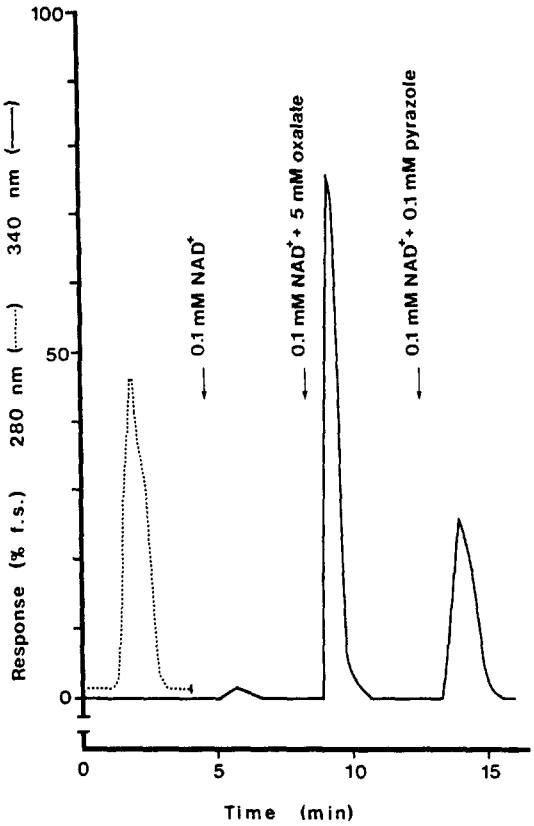


Figure 1. Separation of bovine serum albumin (25 µg), beef heart lactate dehydrogenase (5 µg) and horse liver alcohol dehydrogenase (25 µg) on N^6-(6-aminohexyl)-AMP-silica with HPLAC-technique. The AMP-analogue was coupled to tresyl chloride activated LiChrosorb Diol as described in Methods. The sample (450 µl) was applied on a column (0.5x10 cm) containing 29 µmol AMP-analogue/g dry support. The flow rate was 1 ml/min which gave a pressure drop over the column of 7 MPa. The column effluent (0.05 M sodium phosphate, pH 7.5) was monitored at 280 nm and then mixed on-line with assay reagents (13) for dehydrogenases and monitored at 340 nm. The experiment was done at room temperature.

navalin A immobilized on tresyl agarose was also demonstrated. On application of commercially available peroxidase the column was easily washed free of contaminating proteins or peptides and the bound peroxidase could completely be eluted by addition of 0.11 M mannose to the buffer. The obtained A_{403}/A_{280} ratio was 3.15, which corresponded well with the literature data for pure peroxidase (10).

To avoid possible non-specific binding to the gel, we have found that excess tresyl groups on the support can easily be removed by treating the affinity gel with 0.1 M Tris buffer, a strong nucleophile, at pH 7.5, 4 ^{o}C and for 30 min.

High Performance Liquid Affinity Chromatography (HPLAC). The results of a HPLAC experiment on N^6-(6-aminohexyl)-AMP bound to diol-silica (LiChrosorb Diol) are shown in Figure 1. As seen the biospecificity of this general ligand is well conserved in analogy to previous findings with this ligand bound to CNBr activated Sepharose (11). No albumin was bound to the gel as 100 % appeared in the void volume and 80 % of added lactate dehydrogenase and alcohol dehydrogenase were recovered with short pulses of NAD^+ plus oxalate and NAD^+ plus pyrazole, respectively.

CONCLUSION

We have found tresyl chloride to be an excellent alternative to tosyl chloride for the activation of various hydroxyl group carrying supports, being better suited for the immobilization of pH-sensitive ligands and proteins. Activation of the support is rapid, leads to predictable and high degrees of substitution with tresyl groups and the activated gel is stable when stored even in aqueous media. In contrast, the widely applied CNBr activated supports rapidly loose their active groups in aqueous media. With regards to possible leakage it was found that on storage of [^{125}I] albumin bound to tresyl chloride activated agarose (0.8 mmol tresyl groups/g dry weight) in 0.2 M Tris, pH 7.8 at 35 ^{o}C for 48 h, only a few per cent of the protein leaked off, whereas albumin bound to CNBr activated agarose (0.95 mmol cyanate groups/g dry weight) showed a loss of 20 %. This is of importance as leakage of proteins bound to CNBr activated gels in buffers containing amines or proteins is a well recognized problem (12). The coupling capacity at pH 7.5, 4 ^{o}C and overnight appears, as tested with albumin and trypsin inhibitor, to be higher for tresyl chloride than for CNBr treated gels containing the same amounts of active groups (82 and 70 % as compared to 55 and 60 %, respectively).

The activation method described is well suited for coupling of ligands and proteins used in affinity chromatography and should be especially useful for the preparation of immunosorbents as leakage of immobilized antibodies can be a major problem with the alternative techniques at hand. The ease of direct

activation of sorbents applicable to high performance liquid affinity chromatography constitutes an additional advantage.

ACKNOWLEDGEMENTS

We wish to thank the Swedish Natural Science Research Council for their support and Dr P.O. Larsson for helping us running the HPLAC experiment.

REFERENCES

1. Nilsson, K. and Mosbach, K. (1980) Eur. J. Biochem. 112, 397-402.
2. Nilsson, K., Norrlöw, O. and Mosbach, K. (1981) Acta Chem. Scand. B 35, 19-27.
3. Mosbach, K. and Nilsson, K. (1980) Patent pending.
4. Crossland, R.K., Wells, W.E. and Shiner, Jr., V.J. (1971) J. Amer. Chem. Soc. 93, 4217-4219.
5. Ohlson, S., Hansson, L., Larsson, P.O. and Mosbach, K. (1978) FEBS Lett. 93, 5-9.
6. Reeber, A. and Gombos, G. (1976) Concanavalin A as a tool, pp. 400-407, John Wiley & Sons, London.
7. Decker, L.A. (ed.) (1977) Worthington Enzyme Manual, pp. 90-93, Worthington Biochemical Corporation, Freehold, New Yersey.
8. March, S.C., Parikh, I. and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149-152.
9. Kohn, J. and Wilchek, M. (1978) Biochem. Biophys. Res. Commun. 84, 7-14.
10. Theorell, H. and Maehly, A.C. (1950) Acta Chem. Scand. 4, 422-434.
11. Mosbach, K. (1978) Advan. Enzymol. 46, 205-279.
12. Wilchek, M., Oka, T. and Topper, Y.J. (1975) Proc. Nat. Acad. Sci. 72, 1055-1058.
13. Larsson, P.O., Glad, M., Hansson, L., Månsson, M.O., Ohlson, S. and Mosbach, K. (1982) Advan. Chrom., in press.