IMMOBILIZATION OF ENZYMES ON ALDEHYDIC MATRICES BY REDUCTIVE ALKYLATION*

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SUMMARY. We report here a convenient and inexpensive method of attaching enzymes to solid supports which contain diols. Dextran coated porous glass, Sepharose and glass coated with a glyceryl silane were oxidized with $NaIO_4$. Trypsin, carboxypeptidase A, and carboxypeptidase B were bound to the oxidized supports by treatment with $NaBH_4$. The pH dependence of the coupling reaction and loss of lysine in bound trypsin indicate that the immobilization occurs via reductive alkylation. The bound enzymes display good catalytic activity against synthetic substrates and proteins.

Reductive alkylation is a convenient and gentle method to specifically introduce alkyl groups into proteins at ϵ-amino groups of lysine (1-4). Schiff's bases formed between an aldehyde and the amino group may be reduced with sodium borohydride ($NaBH_4$) or sodium cyanoborohydride. We have used this reaction to couple enzymes to carbohydrate supports which had been oxidized with sodium metaperiodate ($NaIO_4$). Dextran-coated controlled-pore glass (Dextran-CPG), Glycophase-CPG, and Sepharose are neutral, hydrophilic supports with a highly porous structure. The Glycophase-CPG is porous glass coated with a glyceryl silane: Glass$\equiv$ $Si(CH_2)_3OCH_2CHOHCH_2OH$.

One goal of our work is to develop a method to activate neutral carbohydrate carriers without the introduction of charge. A neutral support is ad-

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† Abbreviations used: CPG-controlled-pore glass; Bz-L-argOEt, N-α-benzoyl-L-arginine ethylester; Hip-L-phe, hippuryl-L-phenylalanine: Hip-L-arg, hippuryl-L-arginine.

vantageous in that the pH optimum of the enzyme should not be changed in cases where no diffusional limitations occur (5-6). Also, neither substrate nor product should adsorb to a neutral support. Activation of carbohydrate supports is commonly done with cyanogen bromide (7-8). Even with recent improvements (9) in this activation technique, our method seems preferable. Neither unpleasant nor expensive reagents are involved and the pH of the coupling reaction is lower. Moreover, the dimensional stability of the carbohydrate-glass carriers, under a wide variety of operating conditions, is a considerable advantage in applications of bound enzymes and affinity chromatography.

MATERIALS AND METHODS

Glycophase-CPG(550 Å, lot 500) and Dextran-CPG (550 Å, lot 100) were obtained from the Pierce Chemical Co. Sepharose-4B was purchased from Pharmacia. Trypsin (EC 3.4.4.4, lot 2RL2DA) and carboxypeptidase B (EC-3.4.2.2, lot 54A345) were supplied by Worthington. Carboxypeptidase A was the product of Miles-Seravac (EC 3.4.2.1., batch 5-B). Zinc-free insulin was prepared by the method of Carpenter (10). N,N-dimethyl BSA was prepared as described by Lin, et al. (11).

Oxidation of the Supports. Glycophase-CPG (1g) was placed in a 20 ml vial with 10 ml of $NaIO_4$ solution (6mM). A vacuum was applied for about 2 min to remove air from within the matrix. The glass was rotated slowly on a constant torque stirrer for 1 hr at 25°. Dextran-CPG and Sepharose (1 ml) were oxidized in the same way but with 0.1 M $NaIO_4$. The materials were washed with 200 ml of distilled water.

Enzyme Coupling. For trypsin immobilization the buffers were 0.1 M borate (pH 8.5 and 8.0) and 0.1 M HEPES (pH 7.5 and 7.0); both buffers contained 1mM benzamidine. Carboxypeptidase A and carboxypeptidase B were coupled at pH 8.5 in 0.1 M borate buffer, 1 M NaCl. Enzyme solution (5ml, 1mg/ml) and 150 mg of oxidized glass or 1 ml of oxidized Sepharose were rotated in a 20 ml vial at 4°. At timed intervals aliquots were withdrawn and assayed. At t = 20 min and t = 40 min $NaBH_4$ ($\sim$ 0.5 mg) was added. The reaction was terminated after 1 hr and the bound enzymes were washed with 200 ml of distilled water, 200 ml of 2M KCl, and an additional 200 ml of distilled water. The amount of protein bound to the supports was determined as previously described (13).

Enzyme Assays. The catalytic activities of trypsin, carboxypeptidase A, and carboxypeptidase B were determined with B_Z-L-arg-OEt, Hip-L-phe, and Hip-L-arg respectively. For the bound enzymes assays were performed in columns at a flow rate of 2.3 ml/min. The free volume, V was determined with a 1 mM tryptophan solution under conditions of assay (13). The appearance of product as a result of passage of substrate solution through the col-

umn was determined by measuring the absorbance (254 nm)of the effluent with the feed substrate solution as reference.

Digestion of N, N-dimethyl BSA by the various bound trypsin derivatives was followed by treating the column effluent with the ninhydrin reagent of Moore (14). Conditions for proteolysis were as follows: 0.1 M N-ethyl morpholine-acetate buffer pH8,. 025 M $CaCl_2$; protein, 1 mg/ml; 37^0; flow-rate, 0.23 ml/min. After about 1 hr of column operation, 1 ml of effluent was collected and mixed with 1 ml of ninhydrin reagent. Samples were boiled for 10 min and diluted to 5 ml with 50%ethanol. The absorbance (570 nm)of the samples was determined. Complete digestion was accomplished by recirculating the protein solution through a column of Dextran-CPG-trypsin. The column was prepared by mixing 50 mg of the damp enzyme conjugate with 750 mg of damp unmodified Dextran-CPG. The column diameter was 0.8 cm.

RESULTS AND DISCUSSION

The time courses for the immobilization of trypsin on oxidized Glycophase -CPG, Dextran-CPG and Sepharose at pH 8.5 are shown in Figure 1. The initial rapid depletion of enzyme from solution may be ascribed to Schiff's base formation between the support and enzyme, adsorption in the case of Sepharose and Glycophase-CPG, and a slight dilution; the activity at t = 0 was determined before addition of the damp carrier. Control experiments were per-

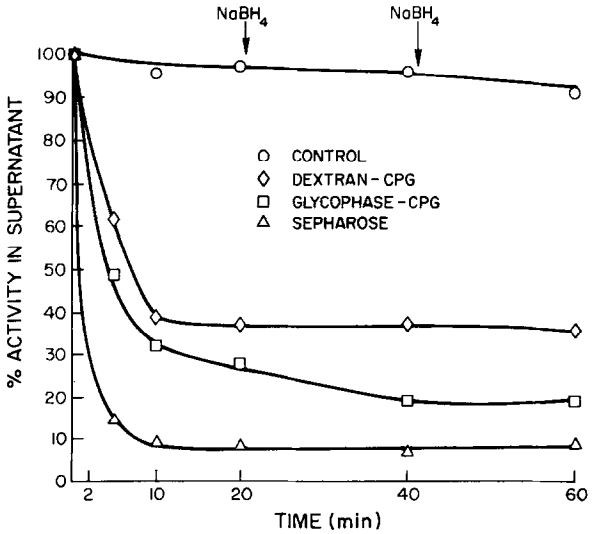

Figure 1. Time course of trypsin attachment of periodate-oxidized derivatives of Glycophase-G CPG, Dextran-coated CPG and Sepharose. The coupling was done at 4^0 and pH 8.5.

formed with unoxidized supports. The Dextran-CPG adsorbed no trypsin over the pH range of 7.0-8.5. Sepharose adsorbed significant amounts of trypsin at pH 8 and pH 8.5. The enzyme, however, was very loosely bound and could be washed off with 2M KCl. The Glycophase-CPG adsorbed trypsin at pH 8 and 8.5. In this case the enzyme was strongly bound. Treatment of the Glycophase-CPG with $NaBH_4$ or 80% acetic acid reduced the adsorption of trypsin only slightly. No adsorption of trypsin to Glycophase-CPG occurred at pH 7.0 or pH 7.5.

When the solid support was reduced prior to addition of enzyme, no binding occurred. In this experiment oxidized Dextran-CPG was treated with two, 0.5 mg, portions of $NaBH_4$ over a 40 min period. This material, used in a normal coupling experiment as shown in Figure 1, bound no enzyme. This result suggests that any aldehyde groups which are not bonded to the enzyme are reduced to alcohols with $NaBH_4$. [4]

In Figure 2 the pH-dependences of the immobilization reactions are shown. The decrease in amount of enzyme bound with decreasing pH is most probably a result of the instability of $NaBH_4$ and the pH dependence of the Schiff's base formation (eq. 1)

$$\begin{array}{ccc} \rangle\!-\!CHO + {}_2HN\text{-}Prot \rightleftarrows \rangle\!-\!CH = N\text{-}Prot \\ \uparrow\downarrow H^+ \qquad\qquad \downarrow NaBH_4 \\ \overset{\oplus}{H_3}N\text{-}Prot \qquad \rangle\!-\!CH_2\text{-}NH\text{-}Prot \end{array} \quad (1)$$

In the cases of Dextran-CPG and Sepharose it is conceivable that tertiary amines could result from reaction of two adjacent aldehyde groups with the same amino group.

If reaction (1) applies, one would expect a loss in lysine when the bound enzymes are subjected to acid hydrolysis and amino acid analysis (Table 1). Notice that the number of points of attachment correlates with the amount of pro-

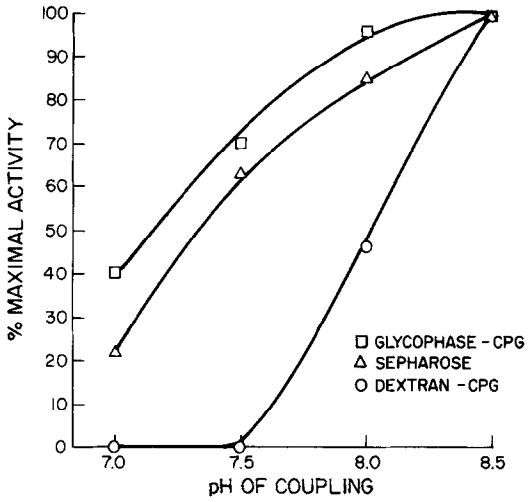

Figure 2. The pH-dependence of the immobilization of trypsin on oxidized Glycophase-CPG, Dextran-CPG, and Sepharose. In each case the activity the enzyme bound at pH 8.5 is taken as 100%.

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tein bound. Also, the amount of protein bound to Dextran-CPG is less than the amount bound to Glycophase-CPG. This is probably due to reduction of the pore size as a result of the dextran coating.

The k_{cat} values shown in Table 1 were calculated from the equation, $\Delta P = k_{cat} E_0 V_f/Q$ in which V_f represents the free volume of the enzyme column and Q is the flow rate. Within experimental error (± 10%) Dextran-CPG-trypsin catalyzes the hydrolysis of Bz-arg-OEt as well as the native trypsin used in these experiments. Complete retention of activity of a bound enzyme has been reported in at least one other case, i.e. aminopeptidase M bound to an arylamine derivative of porous glass(15). The k_{cat} value for Glycophase-CPG-trypsin and Sepharose-trypsin are no doubt reduced by diffusional limitations (16-17). Reduction in the amount of enzyme bound would probably increase these k_{cat} values.

The bound trypsins are also active against protein (Table 1). In this case

Table 1

Trypsin Derivative	Protein bound (Mg/100 mg support)	k_{cat}[a] · sec (B_2-Arg-OEt)	k_{cat}[b] · sec (BSA)	Moles lys lost / moles of trypsin
Dextran-CPG	0.13	14.3	0.45	2.0
Sepharose	0.76	6.6	0.19	4.1
Glycophase-CPG	2.21	3.8	0.04	7.6
Native	—	14	0.60[c]	—

a. Determined with 1mM Bz-Arg-OEt at pH 8.0, 0.025 M $CaCl_2$, 0.05 TRIS, 25^0.
b. Determined with 1 mg/ml N, N dimethyl BSA, 0.1 M, N-ethyl morpholine-acetate, pH 8, 37^0; 0.025 M $CaCl_2$.
c. Estimated considerable curvature was seen in the assays of soluble trypsin with N, N-dimethyl BSA. Lin et al (11) observed this in trypsin assays with other protein substrates.

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also, the catalytic efficiency goes down as enzyme loading increases. Dextran-CPG-trypsin catalyzed the complete digestion of N, N-dimethyl BSA in a 16 hr digest. Complete hydrolysis suggests that the Dextran-CPG-trypsin would be useful in preparing peptides for sequencing. Contamination of the digest by trypsin autolysis products would not be a problem, since the bound enzyme does not autolyze.

Carboxypeptidases A and B have also been immobilized by the method described here. Although an extensive characterization of these derivatives is not complete, they show activity against Hip-L-phe and Hip-L-arg, respectively. Glycophase-CPG-carboxypeptidase A is active against insulin and BSA. In both cases the expected amino acids were released. Bound carboxypeptidase B catalyzes the hydrolysis of polylysine and the aminoethylated B-chain of insulin from which the C-terminal alanine had been removed.

In conclusion, the carbohydrate-glass supports appear very promising for enzyme applications in which quantitive recovery of products is essential. In

addition, these materials are rigid and may be used in columns at high flow rates. Use of the carbohydrate-glass materials with the activation and binding techniques described here should also be valuable in affinity chromatography.

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