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ACRYLIC COPOLYMERS AS MATRICES FOR THE IMMOBILIZATION OF ENZYMES

II THE EFFECT OF A HYDROPHOBIC MICROENVIRONMENT ON ENZYME REACTIONS STUDIED WITH ALCOHOL DEHYDROGENASE IMMOBILIZED TO DIFFERENT ACRYLIC COPOLYMERS

ANN-CHRISTIN JOHANSSON and KLAUS MOSBACH
Biochemical Division, Chemical Centre, University of Lund, Lund (Sweden)
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SUMMARY

The covalent binding of horse liver alcohol dehydrogenase (EC 1 1 1 1) to cross-linked copolymers of acrylamide and of acrylamide–methylacrylate using the glutaraldehyde method is described It was found that on increasing the hydrophobicity of the matrix by copolymerization of methylacrylate with acrylamide, the apparent Michaelis–Menten constant for the more hydrophobic substrate *n*-butanol decreased by a factor of four, whereas that for ethanol was essentially unaffected The assumption that the more hydrophobic matrix will preferentially adsorb the more hydrophobic substrate was substantiated by equilibrium studies using *n*-[^{14}C]butanol The more hydrophobic copolymer (acrylamide–methylacrylate) showed about 6 times higher binding of radioactivity

The immobilized alcohol dehydrogenase preparations lost only 10% of their enzymic activity after storage for a period of three weeks at 4 °C

INTRODUCTION

In the preceeding part (Part I) examples have been given of the preparation of a number of copolymers illustrating the flexibility offered by the combination of various acrylic copolymers In this section (Part II) an example is given of the utilization of such a "tailor-made" approach in the study of the effect of a change in hydrophobicity of the matrix on enzymic activity Whereas mass-transfer and electrostatic interactions between charged substrates and enzyme supports have been thoroughly investigated to determine their effects upon the apparent kinetics of enzyme systems [1–3], studies on possible hydrophobic effects have been neglected However such studies are of value because of the abundance of lipids in biological membranes The influence of hydrophobicity has been studied in the present investigation by varying only one parameter, i e the amount of the hydrophobic component in an acrylic copolymer The enzyme chosen was liver alcohol dehydrogenase (EC 1 1 1 1) known to have a broad substrate spectrum for both hydrophilic and hydrophobic

alcohols as well as for steroids [4] In addition, increasing information is appearing in the literature on the in vivo function of liver alcohol dehydrogenase, e g the oxidation of ω-hydroxylated fatty acids and steroids which is assumed to take place in the lipid-rich microsomal fraction [5]

MATERIALS

Alcohol dehydrogenase (horse liver, 1 6 units/mg), NAD^+ (grade III) and semicarbazide were purchased from Sigma Chem Co (St Louis, Mo , U S A) Ethanol and *n*-butanol, both of analytical grade, and glutaraldehyde were distilled before use [1-^{14}C]Ethanol (59 2 mCi/mole) was obtained from Amersham (Great Britain) and *n*-[1-^{14}C]butanol (12 1 mCi/mole) from New England Nuclear (Boston, Mass , U S A) Acrylamide, methylacrylate, which was distilled before use ,and *N,N'*-methylenebisacrylamide were obtained from Eastman, Org Chem (N Y , U S A) Ammonium persulphate was obtained from Merck (Darmstadt, Germany), and β-dimethylaminopropionitrile from Fluka AG, Buchs SG (Switzerland) Triton X-100 and toluene (scintillation grade) were obtained from BDH Chemicals, Ltd (Poole, U K) 2,5-Diphenyloxazole and 1,4-bis(2-(4-methyl-5-phenyloxazolyl))benzene were purchased from Arapahoe Chemicals (Boulder, Colo , U S A)

METHODS

Preparation of polymers of acrylamide and of acrylamide–methylacrylate (75 25, w/w)

5 7 g monomer (5 7 g acrylamide or 4 28 g acrylamide and 1 42 g methylacrylate) and 0 3 g *N,N'*-methylenebisacrylamide were dissolved in 0 05 M Tris–HCl buffer (pH 7 3) to a final volume of 59 ml The catalyst system, which consisted of 0 5 ml β-dimethylaminopropionitrile and 0 5 ml ammonium persulphate solution (0 1 g/ml buffer), was added to the monomer solution The polymerization was allowed to proceed for 2 h at room temperature in a beaker, after which time the gel block was cut into 1-mm slides and then pressed through a net in order to get particles of roughly 1 mm diameter The polymer was thoroughly washed in 0 5 M NaCl and in distilled water prior to freeze-drying

Covalent coupling of liver alcohol dehydrogenase to the acrylic copolymers

Both copolymer preparations (300 mg dry weight) were activated for 14 h at 40 °C with a 6% solution of glutaraldehyde in 0 2 M phosphate buffer (pH 7 4) The activated gels were thoroughly washed for one day with several aliquots of ice-cold distilled water Then 3–4 mg of the enzyme were added and the mixture was left to couple for 12 h at 4 °C in 0 1 M phosphate buffer (pH 7 4) The enzyme derivatives were washed several times with 0 1 M $NaHCO_3$ and finally with 0 1 M phosphate buffer (pH 8 0)

Enzyme assay

The activity of liver alcohol dehydrogenase was assayed spectrophotometrically by following the formation of NADH at 340 nm The assay mixture (final volume 8 ml), made up in 0 1 M phosphate buffer (pH 8 0), contained 18 μmoles NAD^+, 600 μmoles semicarbazide and varying amounts of the substrate, ethanol or

n-butanol 15 mg dry polymer, carrying 10 munits of activity were used for all assays After equilibration for 10 min at 25 °C the reaction was started by addition of the alcohol The reaction solution was pumped from the incubation mixture, stirred at 150 rev /min in a conical flask, through a flow cuvette and back again to the enzyme suspension A net filter prevented the gel from entering the flow system

Adsorption of [1-^{14}C]ethanol and n-[1-^{14}C]butanol to the acrylic copolymers

Each copolymer (150 mg dry weight), treated with glutaraldehyde in the same way as those containing the enzyme, was swollen in phosphate buffer (pH 7 4), after which labelled alcohol was added The final volume of 8 ml contained either substrates at a concentration equal to their respective K_m values, i e 0 3 mM ethanol or 0 1 mM *n*-butanol Equilibration proceeded for 2 h at 25 °C The radioactivity in 1 ml of the supernatant solution, dissolved in 10 ml toluene–chloroform solution (2 1, v/v), containing 55 mg 2,5-diphenyloxazole and 1 mg 1,4-bis(2-(4-methyl-5-phenyloxazolyl))benzene, was measured in a liquid scintillation counter, Nuclear-Chicago, Mark I

RESULTS AND DISCUSSION

The bifunctional agent glutaraldehyde has been chosen for the immobilization of liver alcohol dehydrogenase on acrylic copolymers (c f Part I) Although the mode of binding involved in the glutaraldehyde method is not yet fully understood, it has been suggested that glutaraldehyde, when used in excess, reacts with the free amide groups present in polyacrylamide to give a modified support that contains aldehyde groups available for coupling with enzymes [6] Polyacrylamide, as well as the copolymer consisting of acrylamide and the methyl ester of acrylic acid, were prepared by bulk polymerization followed by physical fragmentation Bead polymerization was not used in this case because the solubility of the hydrophobic monomer, methylacrylate, in the hydrophobic phase would lead to polymers of ill-defined composition Both the polyacrylamide and the copolymer preparation of acrylamide–methylacrylate (typical ratio, 75 25, w/w) showed almost the same enzymic activity of 0 7 unit/g dry polymer Similarly, the degree of swelling in aqueous solution of both preparations was practically identical, differing only by about 5% Thus, the main difference between the two types of preparations seems to be the degree of hydrophobicity whereas the cross-linking density appears to be the same This is substantiated by the results from equilibrium studies using labelled *n*-butanol, which showed increased adsorption of the more hydrophobic substrate *n*-butanol to the hydrophobic polymer by a factor of six, compared to the outer solution (Table I) No such preferential adsorption was found with the polyacrylamide gel Ethanol, however, showed almost identical partition in the two polymers The "enrichment factors" given in Table I have been obtained from the difference in radioactivity found in the supernatant solution before and after addition of the polymer, and have been calculated on the basis of the volume (150 μl) occupied by the dry polymer If the calculations were made on the basis of the polymer in a swollen state and thereby in a larger volume (approximately ten times) the "enrichment factor" will be less than six The actual concentration of *n*-butanol in the hydrophobic surroundings of the enzyme cannot be determined but is supposed to be highest in the immediate microenviron-

TABLE I

THE INFLUENCE OF THE DEGREE OF HYDROPHOBICITY OF THE MATRIX ON THE MICHAELIS–MENTEN CONSTANTS, K_m, ($K_{m(app)}$ FOR IMMOBILIZED ENZYME) OF ALCOHOL DEHYDROGENASE FOR ETHANOL AND *n*-BUTANOL AT pH 8 0

The NAD^+ concentration was kept constant at 2 mM and the alcohol concentration was varied from 0 16 to 5 0 mM for ethanol and 16 to 590 μM for *n*-butanol The adsorption of the labelled substrate, ethanol (300 μM, 8 ml total volume) or *n*-butanol (100 μM, 8 ml total volume) to the glutaraldehyde treated copolymers after 2 h equilibration was determined by measuring the amount of radioactive alcohol remaining in the supernatant Three independent equilibrium studies were carried out with identical results ($\pm$0 8%) The figures, given as enrichment of adsorbed alcohol, have been calculated assuming the adsorption to be restricted to the volume occupied by dry polymer (see text)

Preparation	K_m (mM)		Radioactivity found in supernatant after equilibration			
			[1-^{14}C]ethanol		*n*-[1-^{14}C]butanol	
	Ethanol	*n*-Butanol	cpm/ml supernatant	calc enrichment to copolymer (-fold)	cpm/ml supernatant	calc enrichment to copolymer (-fold)
Soluble alcohol dehydrogenase	0 30	0 09	–	–	–	–
Alcohol dehydrogenase bound to polymer of acrylamide (100%)	0 57	0 16	11 140	(1)	37 575	(1)
Alcohol dehydrogenase bound to copolymer of acrylamide–methylacrylate (75 25, (w/w))	0 54	0 04	11 210	0 96	33 350	5 9

ment of the enzyme, decreasing gradually with increasing distance from the polymer chains The measured decrease in the K_m values for the *n*-butanol by a factor of four as the enzyme is immobilized to the hydrophobic matrix, correlates with the observed preferential adsorption of *n*-butanol to the matrix The apparent K_m of the two immobilized enzyme preparations for ethanol are almost identical The fact that the K_m values of our insolubilized enzyme preparations are higher than those of the soluble enzyme, is in line with a great number of observations on increased K_m for immobilized enzymes, most likely caused by diffusion limitations exerted on the substrate by the matrix [7] The oxidation of alcohol was measured in all the assays carried out with the cofactor NAD^+ being present in a non-rate limiting concentration, i e 200 times higher than K_m measured for the native enzyme The stability of both types of immobilized alcohol dehydrogenase preparations was excellent with only 10% loss of activity over a period of three weeks at 4 °C, whereas soluble enzyme under corresponding conditions lost 50% of its activity

The four-fold decrease in apparent K_m for the hydrophobic substrate *n*-butanol on the more hydrophobic gel has been obtained repeatedly In line with our studies is a report on an observed 3-fold decrease in the $K_{i(app)}$ for the β-fructofuranosidase inhibitor aniline, when the enzyme is bound to the hydrophobic support polyaminostyrene, whereas no change was found for the hydrophilic inhibitor tris(hydroxymethyl)amino methane [8] Also of interest are similar effects described in a study on

the elution of various compounds from polymers, used as model for the clinical application as depot preparations [9] Here it was found that the more hydrophobic benzoic acid will, when entrapped in a lipophilic gel (copolymer acrylamide–methylmethacrylate (10 90, w/w)), elute far slower than the more hydrophilic phenylacetic acid We feel that studies of the type described in the present paper are of value for two reasons They point to the possibility of tailor-making enzyme supports to suit specific purposes, such as preparations of "substrate-attracting" gels which will permit enzymic reactions to be carried out more efficiently at a rather low total substrate concentration The point made here is illustrated in Fig 1, showing Michaelis–Menten plots of the enzyme, immobilized to both types of copolymers, for the substrate *n*-butanol At a concentration of 50 μM, for example, the rate of reaction was found to be about 75% higher with the more hydrophobic matrix (acrylamide–methylacrylate), compared to that of polyacrylamide

Furthermore, such studies demonstrate that great care should be taken when attempting to understand metabolism in general and its regulation in particular, based on kinetic constants obtained from the study of solubilized enzymes normally present n lipid containing structures such as membranes

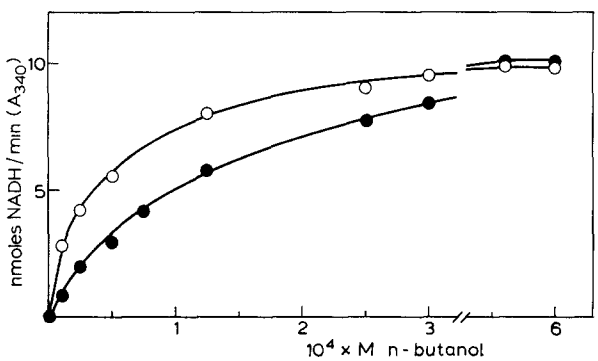

Fig 1 The curves shown are Michaelis–Menten plots of liver alcohol dehydrogenase immobilized to polyacrylamide (●—●) and to acrylamide–methylacrylate polymer (○—○) using *n*-butanol as substrate at pH 8 0 The NAD^+ concentration was 2 mM

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