Synthesis and properties of expanded poly(acryloyl morpholine)/carbonic anhydrase conjugates with catalytic activity in aqueous-organic media

R. Epton, M. E. Hobson and G. Marr
Department of Physical Sciences, The Polytechnic, Wolverhampton, WV1 1LY, UK
(Received 1 July 1977)

The synthesis of an activated, expanded poly(acryloyl morpholine) gel network suitable for enzyme immobilization is described. Carbonic anhydrase conjugates were prepared by covalent coupling via an acid azide method. The enzyme activity of the conjugates and the native enzyme was studied in water, dimethylformamide–water, ethanediol–water, tetrahydrofuran–water and methanol–water. The bound enzyme exhibited enhanced activity relative to the native enzyme in all the solvents used and the bound enzyme showed a high resistance to denaturation on storage.

INTRODUCTION

To date there has been sporadic interest in the utilization of enzymes in aqueous–organic solvent systems. Cremonesi *et al.*[1,2] and Lugaro *et al.*[3] studied enzyme reactions in a two-phase system. The aim was to carry out the enzyme catalysed reaction at the aqueous–organic interface of droplets in an emulsion. This facilitated the study of enzyme reactions on substrates which were sparingly soluble in water. Work on enzymic reactions in aqueous–organic solvent systems at sub-zero temperatures has been employed by various workers in the detection of enzyme–substrate complexes[4–11], especially the intermediates formed by chymotrypsin[4,5,7,12], horseradish peroxidase[9,11] and more recently elastase[7,8].

A major problem in investigating an enzyme in non-aqueous media is its solubility. Often, it is difficult to ensure adequate dissolution of a given enzyme and to prevent precipitation or oligomerization of enzyme molecules[12].

In the present study, we have approached the solubility problem by attaching the enzyme molecule to a purpose synthesized polymer network which will undergo gelation in both water and organic solvents. The enzyme was coupled to the aqueous gel network and the solvent was then changed to an aqueous–organic mixture which was under study, without any risk of precipitation.

EXPERIMENTAL

Synthesis of the copolymer of acryloyl morpholine/N-acryloyl-N'-t-butoxycarbonyl hydrazine (molar ratio 10/1) in bead form

The apparatus used for bead polymerization has previously been described by Epton *et al.*[13]. Acryloyl morpholine (4.6 g, 0.033 mol) (Koch-Light Ltd, UK) and *N*-acryloyl-*N'*-t butoxycarbonylhydrazine[14] (0.6 g, 0.0033 mol) were dissolved in water to form a 50% w/v solution. An aliquot (0.1 cm^3) of 2.5% (w/v) ammonium persulphate solution was added to the deoxygenated monomer solution which was then dispersed as droplets in oxygen-free liquid paraffin, (150 cm^3, η^{20} = 3.5–4.0 N sec/m^2), containing 3% Span 85 (Koch-Light Ltd, UK). The reaction mixture was stirred gently, under a N_2 atmosphere for 24 h to allow complete polymerization, after which the gel beads were equilibrated with light petroleum (b.p. 60°–80°C), ethanol and water.

Activation of copolymer beads

Aliquots (2 cm^3) of the centrifuged polymer suspension were mixed with 2 M HCl (5 cm^3) and stirred at 25°C. Samples were removed after 1 h and 2.5 h and assayed for acid hydrazide, by the method of Inman and Dintzis[15].

Estimation of solvent regain in the gel

The gravimetric method of Holding[16] was used to estimate the solvent regain of the gel prior to, and after acid hydrolysis. Estimations were achieved after the incubation times of 1, 2.5 and 48 h. Air was sucked through a bed of the gel to remove external solvent and a sample of beads from below the surface was transferred to a preweighed microscope slide. The gel was dried to constant weight and the solvent regain (S_R) determined by the equation:

$$S_R = \frac{w_1 - w_2}{w_2}$$

where w_1 is the weight of swollen gel, after removal of external solvent and w_2 is the weight of dry gel.

Preparation of enzyme conjugates

An aliquot of the acid incubated suspension was treated directly with precooled 1 M $NaNO_2$ solution (1 cm^3) at 0°C. After 5 min magnetic stirring, the gel was neutralized with $(C_2H_5)_3N$, equilibrated with the 0.2 M phosphate buffer (pH 7.2), to be used for enzyme coupling, and centrifuged, discarding excess buffer. A 3% w/v solution (2 cm^3) of carbonic anhydrase (E.C. 4.2.1.1.) (Sigma, Cat. No. C7500) was

added and the mixture stirred magnetically at 0°–2°C for 18 h. The enzyme conjugate was washed exhaustively with more coupling buffer, until the discarded washings showed zero carbonic anhydrase activity. The conjugate was stored in the same buffer at 0°–2°C.

Determination of carbonic anhydrase activity

The method of Armstrong *et al.*[17] was adapted. A 0.025% solution of *p*-nitrophenyl acetate, prepared immediately prior to each determination, was used as substrate. Standard aliquots of native enzyme solution (100 mm^3) or enzyme conjugate suspension (33.5 mm^3) were mixed with 50 mM Tris-HCl buffer (pH 7.2) to make a total volume of 2 cm^3. The substrate solution (1 cm^3) was added and the resultant mixture was incubated at 25°C and the change in extinction (348 nm) observed. Whenever the enzyme conjugate was used it was stirred magnetically at constant speed. Prior to extinction measurements the polymer network was removed from the assay mixture by centrifugation and measurements made on the supernatant digest. Immediately after extinction measurements, the digest was reunited with the enzyme conjugate.

Determination of immobilized protein

Each enzyme conjugate was hydrolysed by treatment with 6 M HCl at 110°C for 18 h. The amino-acids produced were separated into groups by paper chromatography and estimated by quantitative ninhydrin assay[18]. The amount of protein present in the conjugates was estimated by reference to a standard graph obtained on chromatographic assay of a hydrolysate of native carbonic anhydrase.

Confirmation of covalent binding of immobilized carbonic anhydrase

Partial enzyme digests were performed, after which the gel matrix was removed from the incubation mixture by centrifugation. Incubation of the centrifuged supernatants was continued to confirm that further enzyme digestion, which would have indicated 'leakage' of enzyme from the conjugate into solution, did not occur.

Organic solvent activity retention

Enzyme activities of the enzyme conjugates and native enzyme were measured in water, dimethylformamide–water, tetrahydrofuran–water, ethanediol–water and methanol–water systems. The organic solvent was introduced into the assay scheme by preparation of the substrate solution in an appropriate aqueous–organic solvent. When organic solvent concentrations were above 33%, a suitable adjustment in the volume and strength of the buffer were performed to maintain the buffer concentration, pH and keep the assay volume constant.

Determination of K_m *and* V

The activities of the enzyme conjugates and native carbonic anhydrase were determined at a series of *p*-nitrophenyl acetate concentrations between 0.75 mM and 6.12 mM at 25°C. Determinations with the enzyme conjugates were performed in various ethanediol–water solvent systems, as well as water. The Lineweaver–Burk[19] graphical method was used to calculate K_m and V.

Stability of the polymer–enzyme conjugates

Thermal stability was determined by incubating samples of the enzyme conjugate and native enzyme in the assay buffer at 60°C. Enzyme activity was determined at intervals for the native enzyme assayed in water and the enzyme conjugate assayed in water and 33% v/v ethanediol–water.

Storage stability for the enzyme conjugate, prepared after 2.5 h acid incubation of the polymer, using the assay procedure previously described was found, after 2 months, to re-

Table 1 Acid hydrazide content and enzyme bound by poly(acryloyl morpholine) copolymer

Incubation time with acid (h)	Solvent regain after acid treatment (ml/g)	Acid hydrazide content* (mmol/g)	Protein content of derived conjugate (mg/g)
1	18.4	0.16	7.3 (Conjugate A)
2.5	22.3	0.35	47.4 (Conjugate B)
48	8.8	Not coupled†	Not coupled

* Estimated by the method of Inman and Dintzis[15].
† Coupling was not attempted owing to diminution of the solvent regain. The solvent regain prior to hydrolysis was 13.0 ml/g

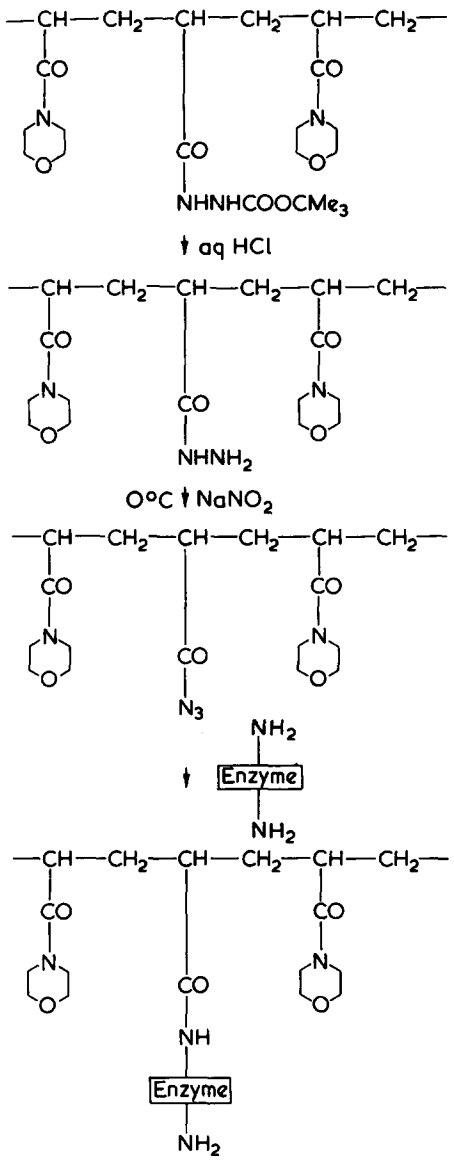

Figure 1 Activation of poly(acryloyl morpholine/*N*-acryloyl-*N'*-t-butoxycarbonyl hydrazine) and subsequent enzyme coupling

Table 2 Enzyme activity of Conjugates A and B and soluble carbonic anhydrase in various aqueous/organic mixtures

Solvent	Organic solvent in water (%)	Activity (%)*		
		Soluble enzyme	Enzyme Conjugate A	Enzyme Conjugate B
H_2O (pure)	0	100	120.0	141.8
Dimethylformamide–H_2O	1.0	107.6	–	177.9
Dimethylformamide–H_2O	2.5	91.0	–	166.4
Dimethylformamide–H_2O	5.0	85.6	–	145.6
Dimethylformamide–H_2O	7.5	69.3	–	95.1
Dimethylformamide–H_2O	10.0	34.8	123.5	88.2
Dimethylformamide–H_2O	20.0	20.2	–	28.5
Dimethylformamide–H_2O	26.6	–	48.9	–
Dimethylformamide–H_2O	33.0	Inactive	34.8	15.0
Tetrahydrofuran–H_2O	1.0	106.9	–	123.1
Tetrahydrofuran–H_2O	2.5	79.3	–	113.8
Tetrahydrofuran–H_2O	3.3	..	94.0	–
Tetrahydrofuran–H_2O	5.0	72.8	–	97.8
Tetrahydrofuran–H_2O	7.5	69.2	–	80.5
Tetrahydrofuran–H_2O	10.0	47.7	91.1	73.2
Tetrahydrofuran–H_2O	20.0	Inactive	–	24.4
Tetrahydrofuran–H_2O	26.6	Inactive	77.4	–
Tetrahydrofuran–H_2O	33.3	Inactive	27.4	10.3
Methanol–H_2O	1.0	92.2	–	108.6
Methanol–H_2O	2.5	67.1	–	86.9
Methanol–H_2O	3.3	–	102.9	–
Methanol–H_2O	5.0	50.1	–	68.1
Methanol–H_2O	7.5	35.6	–	54.3
Methanol–H_2O	10.0	19.5	–	47.1
Methanol–H_2O	13.3	–	82.4	–
Methanol–H_2O	20.0	13.4	–	39.9
Methanol–H_2O	26.6	–	45.7	–
Methanol–H_2O	33.0	Inactive	17.0	19.9
Ethanediol–H_2O	1.0	103.8	–	155.3
Ethanediol–H_2O	2.5	110.3	–	179.6
Ethanediol–H_2O	5.0	115.5	–	145.6
Ethanediol–H_2O	7.5	105.0	–	116.5
Ethanediol–H_2O	10.0	105.7	160.3	129.3
Ethanediol–H_2O	20.0	57.8	–	88.9
Ethanediol–H_2O	26.6	–	121.9	–
Ethanediol–H_2O	33.0	43.7	82.4	53.8
Ethanediol–H_2O	66.0	Inactive	55.0	Inactive
Ethanediol–H_2O	90.0		54.9	–

* Activity expressed as a percentage of the native enzyme assayed in pure aqueous buffer under the conditions of the standard assay

tain 96% of its original activity. The native enzyme in solution retained 94% of its original activity after a similar storage period.

RESULTS AND DISCUSSION

The study of immobilized enzymes in aqueous–organic solvent systems at room temperatures has received little attention. In the present study we have synthesized a covalent, reactive poly(acryloyl morpholine) polymer matrix for use in enzyme conjugate preparation. It was anticipated, from previous work on poly(acryloyl morpholines) that this network would undergo swelling in both water and organic solvents[13]. Traditional enzyme supports, for example cellulose, crosslinked dextrans and crosslinked polyacrylamides swell in water but do not in organic solvents[20].

Our reaction scheme involved bead polymerization of acryloyl morpholine and *N*-acryloyl-*N'*-t-butoxycarbonyl hydrazine molar ratio 10/1. Polymerization was effected at a high enough aqueous concentration of total monomers to ensure spontaneous crosslinking between the copolymer chains. The crosslinking reactions are difficult to define. They may involve initiator mediated α-hydrogen abstraction to give free radical sites which subsequently lead to dimerization of adjacent polymer chains. Alternatively, loss of a β-hydrogen atom from a free radical terminus to give an alkene end-group which may be incorporated subsequently in another copolymer chain is a possibility.

Poly(acryloyl morpholine) beads containing different amounts of acyl hydrazide groups and of varying water regain were obtained by treatment with 2 M HCl. (*Table 1* and *Figure 1*). Prolonged acid treatment of the copolymer beads was harmful insofar as it promoted catastrophic shrinking of the gel. This was probably due to a crosslinking effect:

$$—CO—NHNH_2 \cdot HCl + ——CONHNH_2 \longrightarrow —CO—NHNH—CO—— + NH_2NH_2 \cdot HCl$$

The copolymer in the azide form was coupled with carbonic anhydrase (*Figure 1*). The conjugate derived from copolymers containing 0.16 and 0.35 mmol acid hydrazide/g of dry polymer were designated Conjugate A and Conjugate B, respectively.

In water, both enzyme conjugates show an increased activity as compared to the native enzyme. The practical activity retentions of the enzyme–polymer conjugates and native

enzyme in various organic solvents are shown in *Table 2* and *Figure 2*. Marked rate enhancement was observed in dimethylformamide–water and ethanediol–water for both native and bound enzyme. No such effect was observed in tetrahydrofuran–water and methanol–water. Methanol and monohydric alcohols in general are reported to be inhibitors of carbonic anhydrase[17,21–23]. The difference between ethanediol and monohydric alcohols has been noted previously by Douzou[10], who suggested that the additional hydroxyl group decreased the confirmational denaturing effect in the case of ethanediol.

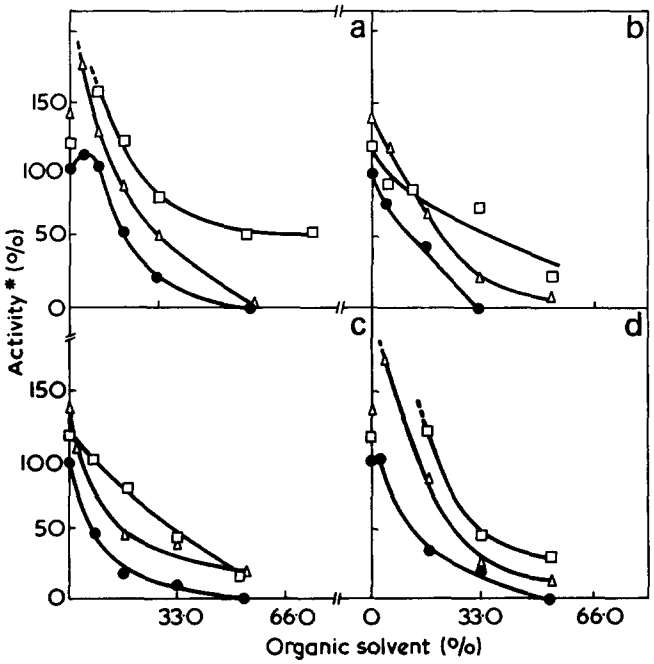

Figure 2 Carbonic anhydrase activity in aqueous/organic solvent mixtures expressed as a percentage of that of the native enzyme in pure aqueous buffer: native carbonic anhydrase (●); Conjugate A (□); Conjugate B (△). (a) ethanediol–water; (b) tetrahydrofuran–water; (c) methanol–water; (d) dimethylformamide–water.
* Activity compared to native enzyme in H_2O

Table 3 K_m and V for copolymer A and B and soluble carbonic anhydrase in water and ethanediol/water mixtures

Preparation	Solvent	K_m (nM)	V (mmol/min mg)
Soluble enzyme	H_2O	4.2	3.1
Conjugate A	H_2O	5.0	5.1
	33% ethanediol	3.7	3.0
	90% ethanediol	3.3	1.26
Conjugate B	H_2O	5.7	13.3
	33% ethanediol	20.0	22.8

Overall, the bound enzyme was more active in pure aqueous buffer than the native enzyme and this is reflected in the increased value of V observed for the conjugates (*Table 3*). It is significant that Conjugate B, which has a much higher protein content than Conjugate A, exhibited the greatest changes in K_m and V both in water and in an ethanediol/water mixture. The less crowded microenvironment of the enzyme in Conjugate A is probably more akin to that in free solution. It is evident that the properties of the bound enzyme are extremely sensitive to the nature of both the solvent composition and the composition of the supporting gel matrix. Investigation of the comparative susceptibility to heat denaturation of the native enzyme in water and the conjugates in both water and in 33% v/v ethanediol suspension (*Table 4*) revealed that the conjugates initially lost activity more rapidly than the native enzyme. However, the conjugates were more stable over an extended time scale.

We have demonstrated that the poly(acryloyl morpholine) gel network is a useful immobilization matrix for the study of enzyme reactions in aqueous–organic solvent mixtures. The matrix confers excellent storage stability on the bound enzyme. Currently, we are investigating the application of this matrix to the study of other enzymes and solvent systems.

ACKNOWLEDGEMENT

The authors wish to thank Koch-Light Laboratories Ltd, for generous support and a studentship (M.E.H.).

REFERENCES

1 Cremonesi, P., Carrea, G., Sportoletti, G. and Antonini, E. *Arch. Biochem. Biophys.* 1973, **159**, 7
2 Cremonesi, P., Carrea, G., Ferrara, L. and Antonini, E. *Eur. J. Biochem.* 1974, **44**, 401
3 Lugaro, G., Carrea, G., Cremonesi, P., Casellato, M. M. and Antonini, E. *Arch. Biochem. Biophys.* 1973, **159**, 1
4 Fink, A. L. *Arch. Biochem. Biophys.* 1973, **155**, 473
5 Fink, A. L. *Biochemistry* 1973, **12**, 1736
6 Fink, A. L. *Biochemistry* 1974, **13**, 277
7 Fink, A. L. and Ahmed, A. I. *Nature* 1976, **263**, 294
8 Alber, T., Petsko, G. A. and Tsernoglou, D. *Nature*, 1976, **263**, 297
9 Douzou, P., Sireix, R. and Travers, F. *Biochemistry* 1970, **66**, 787
10 Douzou, P. *Biochemie*, 1971, **53**, 1135
11 Douzou, P. *Molec. Cell. Biochem.* 1973, **1**, 15

Table 4 Thermal stability of conjugates of carbonic anhydrase and native carbonic anhydrase at 60°C expressed as % of zero time activity

Time (h)	Native enzyme in H_2O	Conjugate A in H_2O	Conjugate B in H_2O	Conjugate A in 33% ethanediol	Conjugate B in 33% ethanediol
0	100	100	100	100	100
1	74.9	50.0	62.1	66.7	–
1.5	–	–	–	–	46.7
3	33.0	18.3	42.1	55.6	–
3.5	–	–	–	–	32.7
5	18.8	14.0	35.0	24.7	–
5.5	–	–	–	–	26.5

12 Bieloki, B. H. and Freed, S. *Biochim. Biophys. Acta*, 1964, **89**, 314
13 Epton, R., Holding, S. R. and McLaren, J. V. *Polymer*, 1976, **17**, 843
14 Barker, S. A., Somers, P. J., Epton, R. and McLaren, J. V. *Carbohydr. Res.* 1970, **14**, 287
15 Inman, J. K. and Dintzis, H. M. *Biochemistry*, 1969, **8**, 4074
16 Holding, S. R. *PhD Thesis* (1975)
17 Armstrong, J., Myers, D. V., Verpoorte, J. A. and Edsall, J. T. *J. Biol. Chem.* 1966, **241**, 5137
18 Epton, R., McLaren, J. V. and Thomas, T. H. *Carbohydr. Res.* 1972, **22**, 301
19 Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* 1934, **56**, 658
20 Holloway, C. *PhD Thesis* (1974)
21 Verpoorte, J. A., Mehta, S. and Edsall, J. T. *J. Biol. Chem.* 1967, **242**, 4221
22 Lindskog, S., Henderson, L. E., Kannan, K. K., Liljan, A., Nyman, P. O. and Strandberg, B. in *'The Enzymes, Volume V'*, (Ed. Boyer, P. D.), Academic Press, New York, 1971, Ch 21
23 Appleton, D. W. and Sarkar, B. *Bioinorg. Chem.* 1975, **4**, 309