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A kinetic study of microencapsulated bovine carbonic anhydrase

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SUMMARY

Recently, semipermeable microcapsules containing enzymes have been prepared and studied as possible therapeutic agents and as models of biological cells. However, detailed reports on the kinetic properties displayed by these insoluble enzyme preparations have been lacking. Therefore, a study on the enzyme kinetics demonstrated by an encapsulated preparation of bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) was carried out. This study revealed that the low activity demonstrated by the microencapsulated enzyme was most likely due to inefficient encapsulation of the enzyme and not to some inherent property of the insoluble derivative.

During the past few years, a great amount of interest has been shown in water-insoluble enzyme derivatives[1,2]. These enzymatic materials are attractive because they can be used in a number of configurations to carry out particular chemical transformations[3-5]. Naturally, a thorough study of the kinetic behavior of such systems is helpful in preparing the most efficient insoluble enzyme derivatives. Unfortunately, the activities displayed by these preparations are usually low compared to the activity of an equal amount of the same enzyme in solution[6,7]. The activity may be decreased by inactivation or conformational changes[8] of the enzyme during the insolubilization process, diffusion and partition considerations, and by other environmental effects related to the chemical nature of the insoluble support[1,9].

The microencapsulation procedure of Chang *et al.*[10] may avoid some of the causes for low activity. In this process, a free-solution enzyme is enclosed in a very thin, semipermeable membrane (approx. 200 Å)[11]. Thus, microcapsules with thin membranes and large pores may minimize the effects of diffusion, partition and perhaps other microenvironmental effects. Even though a number of microencapsulated enzymes have been used in therapeutic applications and as models of biological cells, very little information is available on the kinetic properties of these derivatives, and no detailed kinetic data have been reported for a microencapsulated enzyme. This paper describes the

kinetic properties of such an insolubilized enzyme, bovine carbonic anhydrase, and establishes some of the relationships between microcapsule formulation and the physical and kinetic properties of the encapsulated enzyme.

Bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) was encapsulated by a modification of the Chang procedure[5,10]. A bovine serum albumin solution was substituted for hemolysate and slight changes were made in the concentrations of other components. A mixture of 0.2 ml of carbonic anhydrase solution and 0.9 ml of a 50% bovine serum albumin solution was treated with 0.5 ml of a 0.4 M solution of 1,6-hexanediamine prepared in 0.45 M $NaHCO_3$-Na_2CO_3, pH 9.8. This solution was emulsified by stirring it for 2 min in 10 ml of a chloroform-cyclohexane (1:4, v/v) solution containing 1.5% Span 85. Then 10 ml of a 0.09% sebacoyl chloride solution, prepared in chloroform–cyclohexane (1:4, v/v) was added and the stirring continued for 45 sec. Thereupon, 30 ml of a chloroform-cyclohexane (1:4, v/v) solution were added to the suspension and stirring was continued for 1 min. The resulting mixture was quickly centrifuged, the supernatant liquid discarded and the microcapsules dispersed in 20 ml of a 25% aqueous Tween 20 solution. The Tween 20 was then removed by further washing of the capsules with a 0.9% NaCl solution.

The kinetic constants for free or microencapsulated bovine carbonic anhydrase were typically determined by using an aliquot assay technique and ascertaining the amount of *p*-nitrophenyl acetate hydrolyzed after a 5-min incubation period. Essentially the same kinetic parameters were obtained for enzyme in solution using either the aliquot technique or a continuous spectrophotometric assay with *p*-nitrophenyl acetate as the substrate. The assay mixture consisted of 2.7 ml of a 0.01 M Tris buffer (pH 7.6, 0.09 M NaCl), 0.3 ml of dimethyl sulfoxide, 0.1 ml of the appropriate concentration of substrate in dimethyl sulfoxide, and 0.1 ml of the enzyme in the Tris buffer. Separate experiments showed that this concentration of dimethyl sulfoxide had no effect on the activity of the enzyme. The extinction coefficient for *p*-nitrophenol in the above assay mixture was determined to be 12 700.

The kinetic constants for microencapsulated carbonic anhydrase were determined using the same assay mixture as that given above except that 0.1 ml of a microcapsule preparation was used instead of 0.1 ml of the enzyme solution. The mixture was stirred vigorously for 5 min at room temperature, the capsules were filtered away from the reaction mixture and the absorbance of the supernatant liquid was then read at 400 nm. The appropriate correction was made for nonenzymatic hydrolysis.

In our encapsulations, we found that the concentration of bovine serum albumin in the preparation markedly affected the formation of the microcapsules. In fact, bovine serum albumin solutions up to 50% concentration were necessary for the preparation of capsules suitable for our purposes. Capsules prepared in this manner were larger (70–110 μm) than the capsules Chang *et al.*[10] prepared with hemolysate (35–75 μm). Dye molecules, of a molecular weight of nearly 1000, diffused rapidly from these larger capsules but they never indicated any evidence of protein leakage.

Carbonic anhydrase possesses esterase activity in addition to its ability to hydrate CO_2 and aldehydes. In 1967, Pocker and Stone[12] published a detailed study of the esterase activity of carbonic anhydrase, using *p*-nitrophenyl acetate as substrate. Since these data were available for comparison, and because their system is convenient to use,

we chose to follow the hydrolysis of the same substrate in our kinetic study.

Table I gives the K_m, $v$max and k_{cat} values obtained for microencapsulated preparations containing various amounts of bovine carbonic anhydrase, as well as values for the enzyme in free solution. The K_m values for encapsulated bovine carbonic anhydrase, neglecting the first case in Table I, are only 3–4 times the solution value. Table II gives the specific activity determined for the encapsulated preparations and compares this data to the free-solution value. The values for k_{cat} and specific activity of the encapsulated enzyme preparations are about one-tenth the solution value. These values were calculated on the assumption that all the enzyme used in the preparation was encapsulated and was fully active.

TABLE I

RESULTS FROM THE LINEWEAVER-BURK PLOTS FOR FREE AND MICROENCAPSULATED BOVINE CARBONIC ANHYDRASE CATALYZED HYDROLYSIS OF *p*-NITROPHENYL ACETATE

The kinetic parameters for both the soluble and microencapsulated enzyme were determined using Lineweaver–Burk analysis at substrate concentrations of 3.15 mM, 2.33 mM, 1.55 mM, 0.94 mM and 0.78 mM.

Total amount of bovine carbonic anhydrase encapsulated (mg)	*pH*	*Bovine carbonic anhydrase (μM)**	K_m *(mM)*	v_{max} *(μM·min^{-1})*	k_{cat} *(min^{-1})***
20***	7.6	4.0	5.8	7.7	1.9
45	7.6	9.4	36.0	196.0	21.0
60	7.6	12.5	59.0	380.0	30.3
Bovine carbonic anhydrase concn. in free solution (μg)					
5	7.6	0.052	13.0	10.0	193
25	7.6	0.26	12.8	80.0	308

*Concentration of bovine carbonic anhydrase in assay mixture. In the case of the microcapsules all the enzyme used in the preparation was assumed to be encapsulated and remain active.
**The free-solution values compare favorably with those given by Pocker and Stone[12], if the inhibition by acetonitrile is taken into account.
***Low activities made estimation of the kinetic parameters difficult..

TABLE II

SPECIFIC ACTIVITIES OF FREE AND MICROENCAPSULATED BOVINE CARBONIC ANHYDRASE

Microencapsulated enzyme – total bovine carbonic anhydrase encapsulated (mg)	*Specific activity (μmoles·min^{-1}·mg^{-1})*	*Specific activity of microencapsulated bovine carbonic anhydrase (% of that of free enzyme)**
20**	19.2	0.74
45	280	11
60	316	12

*Specific activity of bovine carbonic anhydrase in solution was 2600 μmoles·min^{-1}·mg^{-1}.
**Low activity of the preparation made the determination of the specific activity difficult.

The very fact that the K_m value for the encapsulated preparation was close to the solution value, while its specific activity was only one-tenth the solution value indicated that much of the enzyme escaped encapsulation, rather than adverse effects of diffusion, partition, conformational changes or interaction of the substrate with the support. In other words, the activity displayed by the capsules may represent encapsulation of only one-tenth the enzyme originally added. The slightly enlarged K_m values obtained for the capsules may be due to the previously discussed considerations operating at a low level, or may simply reflect the recognized difficulty of obtaining kinetic parameters for carbonic anhydrase[12].

The amount of encapsulated enzyme was independently determined using ^{125}I-labeled bovine carbonic anhydrase. This direct method indicated approximately 10% of the labeled bovine carbonic anhydrase was incorporated into the microcapsules. The activity of encapsulated bovine carbonic anhydrase may, therefore, be essentially identical to that shown by the same concentration of enzyme in solution. Preliminary data shows this to be the case since the enzyme activity displayed by the supernatant liquid after rupturing microcapsules was identical to the activity demonstrated by the intact capsules of bovine carbonic anhydrase, and the capsule ghosts showed no activity. Thus, the enzyme in this insoluble system may have the same kinetic properties as the solution enzyme; however, approximately 90% of the enzyme was not encapsulated. Therefore, more efficient microencapsulation techniques should lead to systems having the same kinetic properties as a free solution of enzyme, yet possessing the insoluble characteristics desired.

In summary, a detailed kinetic study showed that the low activity of microencapsulated bovine carbonic anhydrase was due to inefficient encapsulation of the enzyme and not some inherent property of the insoluble derivative.

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REFERENCES

1 I.H. Silman and E. Katchalski, *Annu. Rev. Biochem.*, 35 (1966) 873.
2 S.A. Barker and R. Epton, *Process Biochem.*, August (1970) 14.
3 R.J. Wilson and M.D. Lilly, *Biotechnol. Bioeng.*, 11 (1969) 349.
4 G.G. Guilbault and J.G. Montalvo, *J. Am. Chem. Soc.*, 92 (1970) 2533.
5 T.M.S. Chang, *Science*, 146 (1964) 524.
6 K.P. Wheeler, B.A. Edwards and R. Whitam, *Biochim. Biophys. Acta*, 191 (1969) 187.
7 A.K. Sharp, G. Kay and M.D. Lilly, *Biotechnol. Bioeng.*, 11 (1969) 363.
8 H.D. Brown, S.K. Chattopadhyay and A. Patel, *Biochem. Biophys. Res. Commun.*, 25 (1966) 304.
9 P.J. Sundaram, A. Tweedale and K.J. Laidler, *Can. J. Chem.*, 48 (1970) 1448.
10 T.M.S. Chang, F.C. MacIntosh and S.G. Mason, *Can. J. Physiol. Pharmacol.*, 44 (1966) 115.
11 T.M.S. Chang and M.J. Poznansky, *Biomed. Met. Res.*, 2 (1968) 187.
12 Y. Pocker and J.T. Stone, *Biochemistry*, 6 (1967) 668.