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STUDIES ON ACETYLCHOLINESTERASE AND CHOLINESTERASE COVALENTLY BOUND TO POLYMALEINIC ANHYDRIDE*

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Summary

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and cholinesterase (acylcholine acylhydrolase, EC 3.1.1.8), respectively, were covalently attached to a cross-linked copolymerisate of maleinic anhydride and butanediol-divinylether. Based on the coupling procedure reported by Brümmer et al. (Brümmer, W., Hennrich, N., Klockow, M., Lang, H. and Orth, H.D. (1972) Eur. J. Biochem. 25, 129—135), a simple method is described which requires only 24 h for completion and provides a sufficient yield.

Although a polyanionic carrier was used the K_m and k_2 values as well as the substrate and pH optima of the bound acetylcholinesterase and bound cholinesterase did not differ considerably from the corresponding values of the free enzymes. Bound acetylcholinesterase and to some extent also bound cholinesterase did not lose any enzymatic activity after storage in saline at 4°C for 140 days.

Introduction

Details of the methods commonly used for physical and chemical coupling of enzymes to water-insoluble carrier systems and of the practical applicability of carrier-bound enzymes for analytical and enzyme-kinetic investigations have been reported in some recent publications [1—3].

Due to the well-known high stability and water-insolubility of carrier-bound enzymes, the general availability of carrier-bound cholinesterases would be of considerable significance particularly for the detection and for the assay

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of organophosphorus compounds. Although some methods for the immobilization of cholinesterases by adequate carriers have been described (for references see Baum et al. [4]), only Axen et al. [5] have precisely compared the data obtained for free and for Sepharose-bound cholinesterases. However, the coupling procedure described by these authors is relatively difficult and time consuming.

In the present paper an attempt is reported to evaluate a more simple procedure to couple cholinesterases and to compare the kinetic data of coupled and free esterases. The method finally employed is based upon the procedure of Brümmer et al. [6] for coupling proteinases covalently to a cross-linked copolymerisate of maleinic anhydride and butanediol-divinylether.

Materials and Methods

Acetylcholinesterase, (EC 3.1.1.7), (*Electrophorus electricus*, 1000 units/mg, Biochemica Boehringer, Mannheim) was used in a 1 : 125 mixture with albumin (human albumin, 100% pure, Behring Werke KG, Marburg). Cholinesterase, (EC 3.1.1.8), (horse serum, 4.5 units/mg, substrate acetylcholine) was obtained from Worthington Biochemical Corp., Freehold, N.J. The copolymerisate of maleinic anhydride and butanediol-divinylether, (Polymaleinsäureanhydrid vernetzt, Art. No. 10272) was supplied by Biochemica Merck, Darmstadt.

Coupling procedure

200 mg of the matrix-bound maleinic anhydride were suspended in 10 ml phosphate buffer (50 mM, pH 7.0) and under vigorous stirring passed into a double-jacketed reaction vessel which was kept at 4 °C. After 1 min 100 mg of acetylcholinesterase-albumin and of cholinesterase, respectively dissolved in 10 ml 0.15 M NaCl + 20 mM $MgCl_2$, was added to the suspension of bound maleinic anhydride (carrier:protein = 2 : 1). Immediately after addition of the protein solution, the pH of the suspension was automatically and quickly titrated with 1.0 M NaOH to pH 10.0. Under the conditions applied, the coupling reaction was completed within 1.5 h. At the end of another 1.5 h period, the temperature was increased from 4 °C to 35 °C, which resulted in a transient consumption of NaOH, until the pH was readjusted to 10.0. The automatic titration could be omitted 5 h after the temperature had been raised, but stirring of the suspension was continued for an additional period of 16 h at 35 °C.

Thereafter, the coupling suspension (20 ml) was diluted by 60 ml of a standard solution (containing: 0.15 M NaCl + 20 mM $MgCl_2$) and centrifuged at 3800 × *g* for 20 min (1st washing step). This was followed by 6 further washing steps with each 80 ml of NaCl solution (2.0, 4.0, 2.0 M) and borate buffer (1.0 M, pH 8.5) alternatively. The eighth washing step was performed with 300 ml of the standard solution, which was finally filtered through a D_3-glass filter. The reaction product was removed from the filter and resuspended in 50 ml of standard solution (= stock solution).

Enzymatic activity

The determination of enzymatic activity was carried out by means of the

pH-stat technique under a nitrogen atmosphere (automatic microtitration device equipped with a 0.25 ml burette, Radiometer, Copenhagen). Experiments were usually conducted at pH 8.0 and 32°C. According to the specific activity of bound acetylcholinesterase and bound cholinesterase, different volumes of the stock solution were added up to 2 ml of the standard solution. Acetylcholine iodide, (3 mM) and butyrylcholine iodide, (3 mM) were used as substrates for both the acetylcholinesterase and the cholinesterase. The titrating agent was NaOH (3 mM) freshly prepared from Titrisol®, Merck, Darmstadt.

Inhibition of the enzymes was accomplished by diethyl-*p*-nitrophenylphosphate, purchased from Riedel-de Haen, Seelze, as Pestanal Paraoxone®. The protein content was determined by the modified Kjeldahl method [7]. Each experiment was performed with at least three different coupling batches of the matrix-bound enzymes.

Results

Protein content and specific activity

Under comparable experimental conditions a smaller portion of the acetylcholinesterase—albumin mixture than of the cholinesterase was covalently bound to the carrier. However, for both cholinesterase preparations almost identical proportions of unbound protein could be found in the first three wash solutions. As shown in Table I the bound acetylcholinesterase displayed a considerably higher apparent specific activity than the bound cholinesterase.

Influence of pH, substrate concentration and temperature on the enzyme activity

The pH-, substrate- and temperature optima for the carrier-bound cholinesterases did not differ from the corresponding values of the free enzymes.

In the concentration range of 0.3—60 mM acetylcholine the typical bell-shaped curve of activity against log (concentration) was obtained for the bound acetylcholinesterase. For the bound cholinesterase no definite optimum was attainable with butyrylcholine as substrate.

TABLE I

Binding of acetylcholinesterase and cholinesterase to the carrier bound maleinic anhydride and specific activity of the carrier-bound cholinesterases. Values are shown as means ± S.E.M. PMA, bound maleinic anhydride.

	Acetylcholinesterase—albumin	Cholinesterase
% Protein covalently bound to PMA	7.0	17.3 (± 2.1)
% Unbound protein		
1st wash solution	71.0	71.2
2nd wash solution	2.0	5.0
3rd wash solution	0.5	1.0
Specific activity of		
free enzymes	9.0 (± 1.0)	12.4 (± 0.3)
carrier-bound enzymes	5.7 (± 0.9)	1.3 (± 0.1)

TABLE II

pH-, substrate- and temperature optima of the bound acetylcholinesterase and the bound cholinesterase.

Optimum of	Bound acetylcholinesterase	Bound cholinesterase
pH	8.0—8.5	8.2—8.7
Substrate conc. (mM)	3.0	—
Temperature (°C)	32	45

K_m values

The K_m value for the bound acetylcholinesterase did not differ from the corresponding values reported by Axen et al. [5] (0.166 mM) and by Kröger [8] (0.216 mM) for the free acetylcholinesterase.

Both for the free cholinesterase and for the bound cholinesterase at least two different K_m values can clearly be separated. The K_m values of the bound cholinesterase are significantly higher than those of the free cholinesterase.

k_2 values

The bimolecular rate constants for the interaction of paraoxone with free and carrier-bound cholinesterases were determined according to Aldridge and Davidson [9]:

$$k_2 = \frac{\ln 2}{t_{0.5} \times (I)}$$

As expected about ten-fold higher k_2 values were found for free and bound cholinesterase than for free and bound acetylcholinesterase. The k_2 values of the carrier-bound enzymes were determined to be smaller than those of the free esterases.

Thermostability

50 and 10% of the maximum activity of the bound acetylcholinesterase and 90 and 30% of the maximum activity of the bound cholinesterase were still maintained after an incubation period of 10 min at 50°C and 60°C, respectively.

TABLE III

K_m values of bound acetylcholinesterase and of free and bound cholinesterase. Values expressed as means ± S.E.M.

	Substrate	K_{m1} value	K_{m2} value
Acetylcholinesterase			
bound	acetylcholine (mM)	0.19	—
free		—	—
Cholinesterase			
bound	butyryl-choline (mM)	0.33 (± 0.06)	1.14 (± 0.13)
free		0.24	0.88

TABLE IV

k_2 values, bimolecular rate constants between paraxone and free and carrier-bound acetylcholinesterase and cholinesterase. Values expressed as means ± S.E.M.

	$k_2 = X \cdot 10^5$ ($M^{-1} \cdot min^{-1}$)	
Acetylcholinesterase		
bound	3.05	
free	3.27	
Cholinesterase		
bound	22	(± 1.4)
free	30	(± 1.5)

Stability

After 140 days of storage at 4°C in the stock solution the enzymatic activity of three batches of the bound acetylcholinesterase did not decrease at all. In the same time, two batches of the bound cholinesterase had decreased to 70—75% of the initial activity, whereas the third was without any loss of activity. Under identical conditions the free cholinesterase lost 30% of its initial activity, whereas the free acetylcholinesterase was completely inactive after 21 days.

Discussion

The covalent coupling of acetylcholinesterase and cholinesterase to a copolymerisate of maleinic anhydride and butanedioldivinylether (bound maleinic anhydride) provides the possibility to yield stable enzyme preparations with the least possible alteration of the kinetic properties. Taking into account the polyelectrolyte theory by Goldstein et al. [10] for the polyanionic carrier (bound maleinic anhydride) used and the positive charge of substrates acetylcholine and butyrylcholine we had to expect a shift of the pH optima to higher and of the substrate optima and the K_m values to lower values, respectively. In contrast to the theoretical anticipations identical pH optima and unchanged activity—pH relationships for carrier-bound and free acetylcholinesterase and cholinesterase were found. Moreover, the K_m values of carrier-bound cholinesterase were determined to be even higher than those of the free enzyme. It was therefore to be assumed that the microenvironment of the carrier bound maleinic anhydride, which is negatively charged, reacts neutrally after completion of the coupling reaction. No attempt was made to investigate which step of the method (e.g. increase in temperature from 4 to 35°C 3 h after the start of the coupling reaction, addition of albumin to acetylcholinesterase, contamination of the cholinesterase preparations with unspecific proteins) might have caused neutralization, particularly of the free carboxyl group of the maleinic anhydride moiety. The k_2 values describing the time course of the inhibition of the free and the bound maleinic anhydride-bound cholinesterases by paraoxone did not differ. The result indicates that a significant influence on the inhibition kinetics by the carrier can be excluded. In addition to an im-

proved thermostability known for carrier-bound enzymes, bound acetylcholinesterase and, to some extent, bound cholinesterase showed no loss of activity during the investigated time interval of 140 days. Acetylcholinesterase coupled to glass proved to be stable for only 56 days [4]. Sepharose-bound acetylcholinesterase retained only 70% of its initial activity after 120 days [5]. Starch-included cholinesterase lost 32% of its initial activity in 140 days and cholinesterase immobilized in polyacrylamide gel, 5% in 90 days [11].

In conclusion, the covalent coupling of acetylcholinesterase and cholinesterase to bound maleinic anhydride proved to be a simple and rapid procedure with a comparably good yield. The kinetic data of the acetylcholinesterase and cholinesterase were not significantly modified by the binding to the carrier. The bound maleinic anhydride-bound cholinesterases were remarkably stable, as compared with results obtained with esterases bound to other carrier materials.

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