INSOLUBLE FORM OF KIDNEY ACYLASE I

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Kidney acylase I was covalently bound to a synthetic polymer carrier Enzacryl-AA using the coupling reaction between the diazo-group of the carrier and the reactive groups of aromatic amino acids of the enzyme. In comparison with the free enzyme, the covalently bound acylase has its pH optimum shifted to the alkaline region and it shows a higher temperature stability. The hydrolytic reaction catalyzed by bound acylase I has a lower activation energy than that found for the free enzyme.

Recently, considerable progress has been made in the preparation of insoluble forms of enzymes[1,2]. One of the enzymes treated in this connection was acylase, mainly for practical reasons. Tosa and coworkers[3-7], studying anex-adsorbed, water-insoluble bacterial acylase, showed, in agreement with our results for kidney acylase[8], that it remains active longer and that it has a higher heat stability. Certain limitation of application caused by the nature of the adsorbed enzyme (*e.g.* elution of the enzyme with a higher substrate concentration or greater pH change) defined the line of research leading to the preparation of the covalently bound enzyme.

EXPERIMENTAL

Enzyme isolation. For the preparation of the insoluble derivative of acylase I we used an enzyme obtained from porcine kidneys according to Birnbaum[9]. This was purified further[10] on a column of DEAE-Sephadex A-25 (Pharmacia, Uppsala) by elution with a linear concentration gradient of sodium chloride (0—0·3M) at pH 7·5. After dialysis and freeze-drying, the specific activity of the acylase was 60 units, *i.e.* 10 times greater than that of the enzyme we prepared according to Birnbaum[9]. One unit of acylase activity was defined as an amount of enzyme catalyzing the deacetylation of 1 μmol acetyl-L-methionine per min at 37°C.

The enzyme activity was determined by a modified method of Birnbaum[9]. 50 μl 0·2M sodium phosphate buffer of pH 7·0, 100 μl enzyme (diluted to have substrate hydrolysis of at most 20% at the end of incubation), and 100 μl 0·05M acetyl-D,L-methionine were incubated for 30 min at 37°C. The reaction was stopped by 3 min boiling and the amount of released amino acid was determined in 100 μl of the incubated sample with ninhydrin[11]. The protein concentration was estimated according to Lowry and coworkers[12].

Binding of the enzyme. As carrier we used the commercial preparation by Koch-Light, Enzacryl-AA. When preparing the insoluble derivative we proceeded[13] according to the Koch-Light

Laboratories catalogue KL3. 100 mg Enzacryl-AA was stirred for 15 min at 0—1°C with 5 ml 2M-HCl and with 4 ml 2% $NaNO_2$. The suspension was centrifuged at 0°C and the "diazo-Enzacryl" was quickly washed with 0·05M sodium phosphate buffer at pH 7·0. The suspension of "diazo-Enzacryl" in this buffer was combined with 1 ml of the enzyme (pH made to 7·0). The course of the coupling (for 48 h at 0—2°C with constant stirring was monitored by determining the decrease of activity in the supernatant. After the coupling was terminated, the suspension of the insoluble derivative was washed with 0·05M sodium phosphate buffer at pH 7·0. The resin was mixed with 5 ml 0·05M sodium phosphate buffer and was maintained at 2—3°C. When examining the dependence of binding on the amount of enzyme added, the amount of proteins varied from 1·3 mg/ml reaction mixture to 6·5 mg/ml.

Dependence of activity on pH. To estimate the dependence of activity on pH we chose such concentrations of both the free and bound enzyme which resulted in the same degree of substrate splitting. The enzyme was incubated with substrate in 0·2M sodium phosphate buffer, its pH varying from 5·5 to 8·5.

Temperature dependence of activity of free and bound enzyme. The stability of the free, soluble acylase I was compared with that of the insoluble derivative at 37—80°C. In the experiment, 100 µl solution of the free enzyme or suspension of the insoluble derivative (at a concentration to keep the rate of substrate cleavage approximately equal) was incubated with 50 µl 0·2M sodium phosphate buffer of pH 7·0 for 10—30 min at a given temperature. After cooling to 0°C, the acylase activity was assayed as above.

Determination of the activation energy. The rate constant of the first-order reaction for the deacetylation of acetyl-L-methionine was measured in the temperature range of 15—45°C. In the experiment, 100 µl of the free enzyme solution or 100 µl of the insoluble derivative suspension (at a concentration to keep the reaction rates approximately equal) was incubated with 50 µl 0·2M sodium phosphate buffer for 30 min at the given temperature.

RESULTS AND DISCUSSION

In the used range of enzyme and carrier concentrations, more than 90% of the enzyme was bound to the carrier. At lower protein concentrations a greater specific activity of acylase on the carrier was achieved. Table I shows the overall balance of binding

TABLE I

Results of Binding Kidney Acylase I to Enzacryl AA

Total enzyme activity in reaction, units	Total amount of protein		Total activity of bound enzyme, units	Loss of activity due to binding	
	in reaction, mg	bound to carrier, mg		units	% original activity
75	1·3	1·14	12	53·7	71
150	2·6	2·20	22	105·0	70
375	6·5	5·05	28	263·0	70

after 24 h of reaction, when the binding of the enzyme is practically complete. The data show, at the same time, that due to binding to Enzacryl-AA, some 70% of the enzyme activity is lost. The pH optimum of acetyl-L-methionine hydrolysis is practically identical for both enzyme forms, the optimum being slightly shifted to the alkaline region in the case of the bound form (Fig. 1).

Before determining the reaction rate dependence on temperature for the free and the bound enzyme, the temperature stability of the two enzyme forms was first established. Fig. 2 shows the stability of the bound enzyme to be much higher; at about 60°C, when the free enzyme has lost 80% of its original activity, the bound enzyme still possesses 70–80% of the original activity.

The dependence of the reaction rate on temperature was followed at 10–40°C, *i.e.* in a temperature range where the enzyme is not denaturated. Fig. 3 (an Arrhenius plot) shows that at about 25°C the dependence ceases to be linear and that other factors may play a role here. The values of activation energy indicate that binding of the enzyme to the carrier pronouncedly decreases the activation energy (from 6·7 kcal/mol to 4·7 kcal/mol).

It was shown in a previous communication that kidney acylase I adsorbed on DEAE-cellulose retains its catalytic activity. The limited application of this method

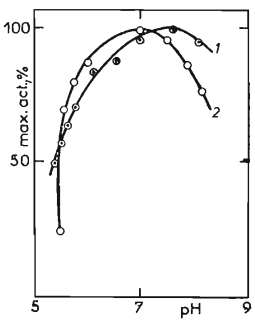

FIG. 1

Dependence of Activity of Free (*2*) and Bound (*1*) Acylase I on pH

The experimental arrangement is described in the text.

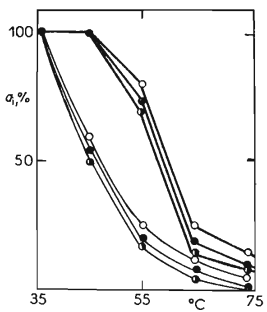

FIG. 2

Temperature Stability of Free and Bound Acylase I

Heavy lines show the bound, light lines the free enzyme. The stability was determined after 10 (○), 20 (●) and 30 (◑) min. Ordinate: % of original activity, abscissa: temperature.

led us to the preparation of a covalently bound enzyme. It was found that acylase cannot be attached to Enzacryl AH by a bond involving the primary amino group of the enzyme[15]. A suitable approach was found in the binding of the tyrosine and histidine residues of acylase molecule to the carrier. However, it appears that the binding of the enzyme to Enzacryl AA probably alters its structure. The loss of 70% activity on binding may indicate that 30% of the bound enzyme is attached to the carrier at a site where the binding or catalytic processes are not affected, whereas the remainder occurs in a region of the enzyme molecule which is necessary for catalytic activity. Alternatively, one may assume that the binding of the enzyme results in such structural changes of the molecular architecture that all molecules suffer a decrease either in affinity or in the reaction rate.

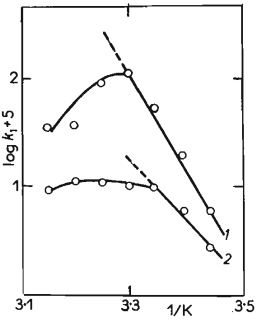

FIG. 3

Dependence of the Rate Constant of the Hydrolytic Reaction on Temperature
1 Free enzyme, 2 bound enzyme. Ordinate, log rate constant; abscissa: $10^3/T$.

Certain comparisons of the properties of covalently bound acylase I may be carried out on the basis of recently published results with the binding of bacterial acylase to halogen-cellulose carriers[14]. Although the enzyme was isolated from a different source, by using a different isolation technique and bound by a different mechanism, it was shown that the pH optimum of the bacterial acylase was also shifted toward the alkaline region and that the activation energy of hydrolysis of acetyl-L-methionine was decreased.

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