IMMUNOCHEMICAL STUDIES WITH
CITRATE-CONDENSING ENZYME

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SUMMARY

Anti-enzyme to citrate-condensing enzyme from pig heart was prepared in rabbits. The interaction of the enzyme and anti-enzyme was not dependent on pH between 6.3 and 8.9 nor was it dependent on temperature at 0° or 37°. The reaction was complete within 10 min of mixing the enzyme and its anti-enzyme. The presence of heat-inactivated enzyme, acetyl-CoA, CoA or citrate did not affect the interaction of active enzyme and anti-enzyme; enzyme inactivated by acetylation retained some reactivity for anti-enzyme. p-Mercuribenzoate-treated enzyme interacted with the anti-enzyme in a manner indistinguishable from untreated enzyme. The enzymes from pig liver and kidney and pigeon heart and breast muscle could not be distinguished from pig-heart enzyme on the basis of their reactivity with the pig-heart anti-enzyme; enzyme from pigeon liver and dog heart, liver and kidney reacted partially whereas that from moth flight muscle and rabbit heart remained completely uninhibited in the presence of pig-heart anti-enzyme. The suggestion is made that the catalytic center and the antigenic determinant may occupy neighboring areas.

INTRODUCTION

Enzyme–anti-enzyme systems provide means for studying the interactions between an antigen and its antibody[1,2] and also between an enzyme and its substrates[3–7]. Similar studies have yielded information regarding enzyme structure[8,9], and the relationship between enzymes from different sources[1–7, 10–13]. The following studies were a preliminary attempt to characterize certain features of the pig-heart citrate-condensing enzyme–rabbit anti-enzyme system.

METHODS AND MATERIALS

Anti-enzyme to citrate-condensing enzyme from pig heart was prepared in New Zealand albino rabbits by injecting them with an enzyme preparation that was approx. 5% pure (see below). 75 mg of the impure enzyme were given as 6 injections over 3 weeks; each dose was injected in part by the subcutaneous, intracutaneous,

intraperitoneal and intravenous routes. One week after the last injection there was no demonstrable anti-enzyme activity in the rabbit serum. A subsequent course of 3 weekly subcutaneous injections of 8 mg of the crude enzyme emulsified in an equal volume of complete Freund's adjuvant (total volume 4 ml) was followed by the appearance of low levels of anti-enzyme. After a similar course of 4 weekly injections, 40 ml of blood were removed from each rabbit. One month later a single booster injection in adjuvant was given and another 40 ml of blood collected. Two months later another course of 4 weekly injections of enzyme in adjuvant were administered and a further 40 ml of blood obtained. The titer of anti-enzyme did not vary appreciably in the sera of individual rabbits over the course of these 3 bleedings. All of the studies to be reported were carried out with the pooled sera from the rabbit with the highest titer; 1 ml of this serum was capable of inactivating 1.2 units[14] of crystalline enzyme (approx. 40 μg). Merthiolate (final concentration of 1 : 10 000) was added to both control and immune sera, and they were stored in 4-ml lots at approx. $-20°$. The anti-enzyme activity remained stable for at least 6 months.

Tube precipitin tests[17] (4–32 μg enzyme and 0.25 ml serum) and OUCHTERLONY double diffusion tests[18] (160 μg enzyme and 0.1 ml serum) were made using 3 times recrystallized enzyme. No specific precipitate could be detected.

The interaction between enzyme and anti-enzyme was studied using the capacity of the immune serum to inhibit enzyme activity. For each determination one tube with normal rabbit serum and another with immune serum were used; the control serum had no anti-enzyme activity. Each tube contained 0.20 ml of diluted serum (1 : 8 with 0.15 M sodium chloride in 0.02 M potassium phosphate (pH 7.4)), and 0.05 ml of enzyme adjusted to the appropriate concentration with 1 : 50 normal rabbit serum. The volume of reactants was increased proportionally when larger total volumes were required. Except where otherwise indicated, the pH of the reaction mixture was 7.3, the temperature was maintained at 0° and the time between mixing and assay did not exceed 5 h. Neither the control nor immune sera contained citrate-condensing enzyme activity.

Enzyme activity was assayed at 340 mμ (see ref. 19) in a Beckman DU spectrophotometer with a Minneapolis Honeywell recorder and a Gilford absorbancy converter and automatic cuvette changer. The activity measured in the presence of immune serum was expressed as a percent of that in the concurrently assayed control; this is referred to as the "percent residual activity". The cuvette chamber was maintained at approx. 25°; the final cuvette temperature was measured with a thermistor and enzyme activity corrected to 25° (see ref. 16).

The change in absorbancy was followed for 10 min and the activity computed on the basis of the first 8-m: interval. As little as $4 \cdot 10^{-5}$ unit of enzyme (approx. 1.2 mμg) could be measured with relative ease.

The preparation of pig-heart citrate-condensing enzyme used in immunizing the rabbits contained malate dehydrogenase[18] and the immune serum (but not the control) was found to have anti-enzyme to pig-heart malate dehydrogenase. Since the assay for citrate-condensing enzyme was a coupled one dependent on malate dehydrogenase, a concentration of malate dehydrogenase was taken that was ten-fold higher than that used for standard assays and greatly exceeded the anti-malate dehydrogenase capacity of the serum. Both the control and immune sera contained

malate dehydrogenase activity; this activity alone would have been sufficient to support the usual coupled assay.

Boehringer malate dehydrogenase and CoA were used and acetyl-CoA was prepared according to the method of SIMON AND SHEMIN[20]. The antigen used for injecting the rabbits, referred to as the 50–70% ammonium sulfate fraction, was prepared as described earlier[16]. Crystalline condensing enzyme was prepared from pig heart either as previously described[16] or by a recent modification of the method. Citrate-condensing enzymes from other tissues were prepared in the following way: the tissue was homogenized for 5–10 min in a Waring Blendor with 4 volumes of 50% ammonium sulfate. The suspension was centrifuged and ammonium sulfate was added to the supernatant solution to 70% saturation. The precipitate was collected and dissolved in a small quantity of 0.02 M potassium phosphate (pH 7.4). This solution was dialyzed overnight against 0.002 M potassium phosphate (pH 7.4). Any precipitate formed during dialysis was removed by centrifugation and the supernatant solution used as a source of condensing enzyme. The crystalline pigeon breast and moth flight muscle enzymes were prepared with methods similar to that used for the pig-heart enzyme.

RESULTS

Time-course of the enzyme–anti-enzyme interaction

Crude enzyme (50–70% ammonium sulphate fraction) and 3 times recrystallized enzyme were added in varying concentrations to control and immune sera (Table I).

TABLE I

TIME-COURSE OF THE ENZYME–ANTI-ENZYME INTERACTION

Conditions at 0° and at pH 7.3

Units added per 0.2 ml 1:8 serum	Percent residual activity								
	6–10 min	1 h	4h	1 days	2 days	4 days	5 days	8 days	10 days
	Crude enzyme								
0.344	92	89	86			85	92		
0.125	60	77	58	53	64	58			62
0.061	49	41	38	35	42		42	34	
0.037	13	12	10	10	1				0
	Recrystallized enzyme								
0.349	91	91	100	74		85	85	81	
0.134	68	70	71	66	64	67			67
0.066	55	35	46	49	44		42	43	
0.038	22	10	22	19	23				13

Within 6–10 min at 0° the reaction was complete. Crude and recrystallized enzyme behaved similarly. The experiment was also repeated with the mixture first at 37° for 30 min and subsequently at 0°. Small differences were obtained which did not exceed the variation inherent in the method.

Effect of centrifugation

Enzyme- anti-enzyme mixtures were assayed before and after centrifuging at 4000 rev./min for 30 min. No decrease was observed in residual enzyme activity.

Standard curve

A plot of all data obtained within 5 h of mixing enzyme and anti-enzyme at 0° (pH 7.3) is shown in Fig. 1. These results were obtained during a 4-month interval and show a reproducibility of ± 15%. The effect of certain variables on the enzyme-anti-enzyme reaction was always compared with this standard curve.
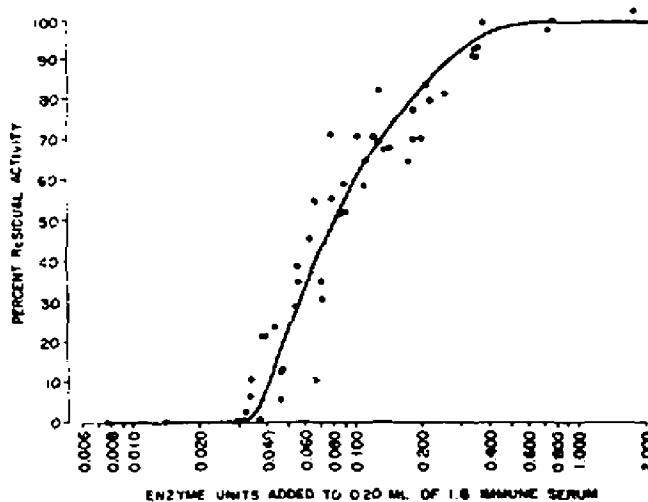

Fig. 1. Interaction of enzyme and anti-enzyme at 0° and pH 7.3. 0.05 ml of enzyme was added to 0.20 ml of 1 : 8 control and immune rabbit serum and the two were assayed concurrently within 5 h of adding enzyme.

Effect of pH

The stability of this enzyme with regard to pH over short intervals of time has been previously described[21]. The enzyme was stable between the pH's of 6.3 and 8.9 over the longer periods of time which this work required. The reaction mixture was adjusted to pH levels within this range by altering the pH of the diluents for the serum and enzyme with an appropriate 0.02 M Tris–citrate or 0.02 M potassium phosphate buffer. At pH 8.9, 6.6 and 6.3 enzyme was inhibited by anti-enzyme to the same extent as at pH 7.3, with no apparent effect of different buffer compositions.

Effect of heat denatured enzyme on the enzyme–anti-enzyme reaction

A solution of citrate-condensing enzyme containing 23.2 units/ml was inactivated at 58° for 5 min. When 0.05 ml of the original solution (before heat inactivation) was added to 0.40 ml of 1 : 8 immune serum, 100% residual activity was found;

this result indicated an excess of enzyme over the quantity of anti-enzyme. Interaction between heat-denatured enzyme and anti-enzyme was assessed by determining if the prior addition of 0.05 ml of this inactivated enzyme to immune serum produced a reduction in the capacity subsequently to inhibit active enzyme. No alteration of this capacity could be detected (Fig. 2). When enzyme was 50% inactivated by heating at 48° for 5 min, the remaining active enzyme reacted with anti-enzyme to the same extent as unheated enzyme.

Interaction of p-mercuribenzoate-treated enzyme with anti-enzyme

p-Mercuribenzoate (3.55 mg) was dissolved in 0.9 ml of water by adding several drops of 5 M potassium hydroxide. The pH was then adjusted to 8 with HCl and the resulting precipitate removed by centrifugation. A preparation of crystalline enzyme was spectrophotometrically titrated at 255 mμ with a 1 : 5 dilution of the p-mercuribenzoate until the increment in absorbancy with each addition of p-mercuribenzoate was equal to that in the blank which contained no free sulfhydryl groups[23]. Crystalline condensing enzyme contains 2–3 free sulfhydryl groups per molecule of enzyme[23]. p-Mercuribenzoate-treated enzyme retained full enzymic activity and reacted with anti-enzyme to the same extent as untreated enzyme (Fig. 2).
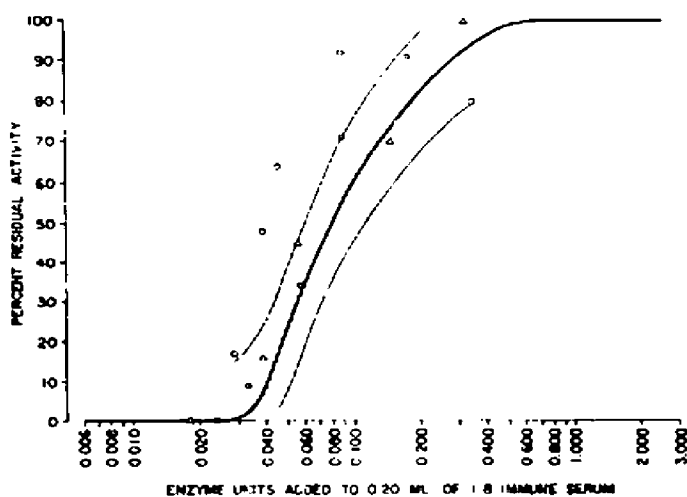

Fig. 2. Reactivity of altered enzyme with anti-enzyme. The data obtained are illustrated in relation to the standard curve and its + 15% limits. Heat inactivated enzyme (□—□): citrate-condensing enzyme was totally inactivated in 5 min at 58°. 0.05 ml of this preparation was added to 0.40 ml of 1 : 8 control and immune serum at 0° (prior to inactivation, when this amount of enzyme was added to 0.40 ml of 1 : 8 immune serum, 100% residual activity could subsequently be measured); following this, 0.05 ml of active enzyme was added. The enzyme activity in the control and immune sera were then measured and the latter expressed as a percent of the former. p-Mercuribenzoate-treated enzyme (△—△): citrate-condensing enzyme retained full activity after complete titration with p-mercuribenzoate. 0.05 ml of treated enzyme was added to 0.20 ml of 1 : 8 control and immune serum and the percent residual activity determined as above. Acetylated enzyme (○—○): citrate-condensing enzyme was totally inactivated by treatment with acetic anhydride at a pH close to neutrality. This preparation was then used in an experiment similar to that described for heat-inactivated enzyme.

Effect of acetylated enzyme on the enzyme–anti-enzyme reaction

Acetic anhydride (0.05 ml) was added to 0.9 ml of water and quickly neutralized with solid potassium bicarbonate. The freshly prepared anhydride was used to acetylate a solution of enzyme containing 23.2 units/ml. The next morning the solution was found to be only slightly acidic and to show no residual enzymic activity. When equal amounts of the acetylated and active enzyme were mixed and allowed to stand for 1 h, no loss of enzyme activity resulted. The interaction between acetylated enzyme and anti-enzyme was then assessed in a similar manner to that described for heat-inactivated enzyme. Addition of the acetylated enzyme produced a small but significant reduction in the capacity of immune serum to inhibit active enzyme subsequently (Fig. 2).

Influence of substrates on the enzyme–anti-enzyme reaction

The enzyme (0.004 μmole or less) in a volume of 0.05 ml was treated for 5 min at room temperature with an equal volume of either acetyl-CoA (0.25 and 0.50 μmoles) or coenzyme A (0.10, 0.25 and 0.50 μmoles) or Tris–citrate (pH 7.4) (1.0 and 2.0 μmoles); 0.4 ml of 1 : 8 serum was then added and enzyme activity assayed. The data in Table II show that the presence of either of these 3 substrates singly, produced no detectable interference with the enzyme–anti-enzyme reaction. These were not tested in pairs or all 3 together; it was not possible to study the effect of oxaloacetate with the assay used in this work.

TABLE II

INFLUENCE OF SUBSTRATES ON THE ENZYME–ANTI-ENZYME REACTION

0.004 μmole or less of enzyme in a volume of 0.05 ml was added to 0.05 ml of substrate, and allowed to stand at room temperature for 5 min. 0.40 ml of 1 : 8 serum was then added and the percent residual activity compared with that found under standard conditions.

Substrate	Units of enzyme present for each 0.30 ml of 1 : 8 serum added	Residual activity found (%)	Predicted from curve (%)
0.25 μmole Acetyl-CoA	0.042	23	15
0.50 μmole Acetyl-CoA	0.073	39	46
0.25 μmole CoA	0.036	3	5
0.50 μmole CoA	0.035	4	4
1.0 μmole citrate	0.045	27	18
2.0 μmoles citrate	0.059	29	34

Reactivity of condensing enzymes from other tissues with rabbit anti-enzyme to pig-heart condensing enzyme

The 30–70% ammonium sulphate fraction from pig kidney or liver extract was added to 1 : 8 rabbit serum under similar conditions to those used for pig-heart enzyme. The data in Table III show that enzyme from these other pig tissues reacted with heart anti-enzyme to the same extent as did the heart enzyme.

The condensing enzymes from pigeon heart and pigeon breast muscle also

TABLE III

REACTIVITY OF CONDENSING ENZYMES FROM OTHER SOURCES WHEN ANTI-ENZYME
TO PIG HEART IS COMPARED WITH THAT OF THE PIG-HEART ENZYME

| Tissue | Enzyme units added to 0.2 ml 1 : 8 serum | Residual activity found (%) | |
		Enzyme from other source + anti-enzyme	Pig-heart enzyme + anti-enzyme
A. 1. Pig liver	0.139	74	72
	0.037	0	5
Pig kidney	0.138	70	72
	0.038	6	7
2. Pigeon heart	0.079	44	50
	0.018	0	0
Pigeon breast	0.072	36	45
	0.019	0	0
B. 1. Pigeon liver	0.017	8	0
	0.009	11	0
	0.005	8	0
2. Dog heart	0.044	99	17
	0.017	82	0
	0.006	59	0
Dog kidney	0.056	89	31
	0.010	75	0
	0.004	73	0
Dog liver	0.031	89	0
	0.008	49	0
	0.004	51	0
3. Moth flight muscle	0.033	92	2
	0.003	103	0
Rabbit heart	0.033	104	2
	0.005	99	0

reacted with the anti-enzyme to pig heart in a manner indistinguishable from pig-heart enzyme; rabbit heart and moth flight-muscle enzymes were not inhibited by the anti-enzyme; enzymes from pigeon liver and dog heart, kidney and liver were only partially inhibited by the anti-enzyme. The latter group appeared to reach a plateau of inhibition which did not change upon reducing the ratio of enzyme to anti-enzyme. When pig-heart enzyme was added to immune serum containing moth flight-muscle enzyme, the pig-heart enzyme was inhibited to the same extent as if the other were not present.

DISCUSSION

The poor antigenic property of pig-heart citrate-condensing enzyme noted here may have resulted from the use of a crude extract in immunizing the rabbits; possibly a more satisfactory response would have been found in the absence of other antigenic stimuli.

The results presented here suggest a close relationship between the catalytic center and the antigenic determinant. The enzyme became totally inactive after being added to an appropriate amount of anti-enzyme; the rapidity of inactivation, the absence of an effect by centrifuging and the lack of demonstrable precipitate minimize the possibility that enzyme was unavailable to substrate through sedimen-

tation from solution in the immunological complex. This suggests that the catalytic center may have become sterically inaccessible to substrate following the interaction of enzyme and anti-enzyme; it is alternatively possible that loss of enzymic activity following union with anti-enzyme may have been due to a resulting distortion of the catalytic center[24–26]. In some other enzyme–anti-enzyme systems, the immunological complex retained at least some enzymic activity[6,14,27–30]. Both the enzymic activity and capacity to react with anti-enzyme were lost in parallel when enzyme was treated with heat; however, this may have been related to alteration of different parts of the molecule. A free sulfhydryl group is not important in either site since p-mercuribenzoate treatment did not affect either enzymic activity or the interaction of enzyme and anti-enzyme. Acetylation of the enzyme destroyed its activity and greatly reduced its capacity to react with anti-enzyme. These observations suggest that the catalytic center and antigenic determinant may be represented in a similar area of the molecular surface, but in an overlapping rather than identical manner.

It would be anticipated from the foregoing that the presence of the substrates might interfere with the enzyme–anti-enzyme reaction. Of other enzyme–anti-enzyme systems in which similar studies were carried out, substrate and cofactors were demonstrated to interfere in some[4–7,9,11,30] and not in others[4–6,9,12,14,15,29,31,32]. Since the individual substrates did not affect the enzyme–anti-enzyme interaction it is possible that the avidity of the enzyme for anti-enzyme is considerably stronger than for substrate, or as found by others[26], that substrate pairs must be present before binding of either occurs. Previous equilibrium dialysis experiments between condensing enzyme and oxaloacetate or [14C]-acetyl-CoA gave no evidence for binding of either material by the enzyme[33].

Pigeon-heart and breast-muscle enzyme were indistinguishable from the pig-heart enzyme by these immunological means. However, the pigeon-breast muscle enzyme behaved quite differently from the pig-heart enzyme in starch-gel electrophoresis, was more soluble in ammonium sulphate, could be eluted from DEAE-cellulose with a lower ionic strength phosphate buffer and was more heat stable[34]. This clearly indicates that the presence of complete immunological identity does not necessarily imply the existence of complete molecular identity[10,15,34].

The plateau of inactivation that occurs with pigeon liver and dog heart, kidney and liver enzymes may be due to (a) the anti-enzyme covering only a fixed proportion of the catalytic centers, (b) more than one form of citrate-condensing enzyme being present in these preparations and not all of them being inhibited by anti-enzyme, or more likely, (c) the dissociation constant between the anti-enzyme and these enzymes being higher.

The findings with rabbit-heart enzyme are of further interest. If in the pig-heart system the catalytic center and antigenic determinant overlap, the failure of the rabbit enzyme to cross react suggests that a comparable determinant is either not present in this molecule or is present but sterically unavailable to the anti-enzyme. The former might be anticipated to be more likely and the rabbit produces anti-enzyme which is specific for some portion of the pig enzyme not present in the rabbit enzyme; however, MARSHALL AND COHEN[31] found that rabbit anti-enzyme to heterologous carbamyl phosphate synthetase inactivated the homologous enzyme.

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