IMMUNOENZYMOLOGICAL EVIDENCE SUGGESTING A CHANGE IN CONFORMATION OF ADENYLIC ACID DEAMINASE AND CREATINE KINASE DURING SUBSTRATE COMBINATION

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ABSTRACT The kinetics of inhibition of 5′-adenylic acid deaminase and creatine-ATP transphosphorylase by their respective antibodies are studied and rate constants of combination are ascertained. It is shown that the single substrate 5′-adenylic acid (AMP) of deaminase "protects" the enzyme against antibody inhibition. However, phosphate, a competitive inhibitor of the highly specific deaminase, enhances combination with antibody. With creatine kinase, however, addition of either of the substrates, alone or in combination with the required magnesium, each of which separately bind to the enzyme, does not prevent inhibition of the enzyme by its antibody. However, the "working" enzyme combined with all substrates is "protected" against antibody inhibition.

Earlier work on the "trophic" influence of the central nervous system on nerve and muscle metabolism (1–3) led to an immunochemical study of muscle proteins which indicated a change in protein structure (4–6) during denervation atrophy. It is not known whether this response of protein configuration to denervation was in the primary structure, similar to the case of the change from fetal to adult hemoglobin, or whether the conformation (secondary structure) of the protein was altered. The use of antibodies to enzymes seemed an excellent method with which to probe the conformational dynamics of enzyme structure as it might occur during enzyme function. If a conformation change, dependent on enzyme function obtains, it would be evidence against the strict isomorphism between structure and function that is usually implicit in theories of enzyme action. Recent reviews (7) on enzymes discuss the evidence for "enzyme dynamics" as a mechanism of enzyme action.

This paper will present evidence of the kinetics of enzyme-antibody combination, in the presence of substrates and competitive inhibitors, from which it may be inferred that a configurational change takes place during enzyme-substrate combination.

METHODS

Inasmuch as this paper is not intended to give in full the details of the immunoenzymology of these enzymes but rather to concentrate on the evidence directly related to the experiments suggesting a change in conformation, the methods will be outlined briefly.

Rabbit antisera were produced against chicken muscle 5′-adenylic acid deaminase (deaminase) as described previously (8). Chicken antiserum to crystalline rabbit muscle creatine–ATP–transphosphorylase[1] (creatine kinase) was produced by repeated intravenous injection of large amounts (100 mg) of the enzyme. Blood was obtained by cardiac puncture; the serum was separated and tested for precipitin and antienzyme activity. In some cases the globulin fraction separated by 25 per cent ethanol was used.

Both deaminase and creatine kinase are completely inhibited upon combination with their respective antibodies (8, 9). The rates of combination in the dilute enzyme-antibody mixtures were followed by pipetting aliquots of the incubating mixture into excess substrate at varying times. This determined the amount of free enzyme. Dilution of the enzyme and antibody by a factor of 10 served to slow greatly the rate of combination. In addition, as will be shown in the results, substrate blocks further combination of the enzyme and antibody.

Deaminase and its antibody were usually incubated at 37 degrees in 0.5 M KCl containing 0.1 M succinate at pH 6.0 and pipetted into 0.0043 M (AMP) 5′ adenylic acid in the same buffer. However, other pH's and ionic strengths were used to incubate the enzyme and antibody. Conditions for creatine kinase were: 30°C, glycine buffer 0.1 M, pH 9, 0.024 M creatine, 0.004 M $MgSO_4$, 0.004 M ATP.

The activity of the enzymes was stopped with cold trichloracetic acid (7 ML of 5 per cent for deaminase and 3 ML of 20 per cent for creatine kinase) and the supernatants analyzed for the products, ammonia by direct nesslerization and phosphate by the Fiske-SubbaRow method (10).

RESULTS

Fig. 1 shows the time course of inhibition. Curves B and E respectively in the figure and its insert are of creatine kinase and deaminase inhibition. The activity for the large figure is expressed in terms of micromoles of creatine phosphate formed and in terms of NH_3 for the insert. Curve A depicts incubation in control serum showing little destruction of activity. Curve C is a plot of 1/activity *versus* time. In this case the activity is converted into moles/liter of free enzyme[2] and the inverse plotted against time. Straight lines were found with both enzymes when the concentrations of the enzyme and antibody were very carefully adjusted to certain levels.

Theoretically this may be treated as a second order reaction in which the rate constant of dissociation of the complex is disregarded, as it is assumed to be very

[1] The author is greatly indebted to Dr. Lafayette Noda for suggestions that aided in crystallizing the enzyme in the initial attempt and for gifts of additional enzyme preparations.

[2] Thus if at time T an aliquot was pipetted into the substrate and found to have $a - x$ units (15) of activity per ml, the moles of free enzyme is calculated by multiplying the dilution factor by ($a - x$ units/a_0 *units*) by the moles of crystalline enzyme added originally. This assumes that only free enzyme has activity.

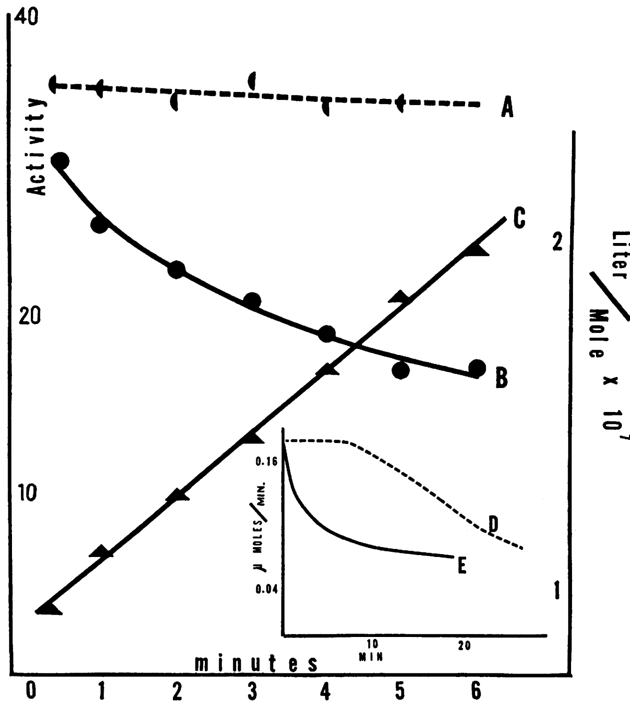

FIGURE 1 Kinetics of inhibition and rate constant of enzyme-antibody formation. Aliquots of the dilute mixture of enzyme and antibody are pipetted at indicated times into substrate.

Main figure—0.004 M ATP, 0.004 M $MgSO_4$, 0.024 M creatine, 0.1 M glycine pH 9.0, 30°C.

Insert figure 0.0043 M AMP, 0.1 M succinate in 0.5 M KCl, pH 6.0, 37°C. Protection by AMP, 0.004 M added simultaneously to enzyme-antibody mixture.

much smaller than the rate constant of association. Even at low enzyme concentrations the contribution of the dissociation term seems negligible (11). At equal concentrations of enzyme and antibody, the equation of this formally irreversible reaction will integrate to

$$\frac{1}{E} = \frac{1}{E_0} + kT$$

and a plot of $1/E$ *versus* T gives a straight line, the slope of which yields the apparent rate constant of combination of the enzyme with the antibody. This is equal to 10^6 to 10^7 liter/mole/min. for creatine kinase. Chicken muscle deaminase and its rabbit antibody give a rate constant of the same order of magnitude if one assumes it has the same molecular weight as the rabbit muscle enzyme, *i.e.*, 3.2×10^5 (12). This assumption may be justified as it has a closely similar Km, 1.43×10^{-3} M (5, 12), and the turnover number of purified, but not crystalline, preparations is

3×10^6 moles/mole/hr. These rate constants are in agreement with the results of Berson and Yalow (13) who have given 10^5 to 10^7 liter/mole/min. as the forward rate constant and 10^9 to 10^{10} liter/mole as the equilibrium constant for the insulin-antibody combination.

When substrate, AMP, is added to deaminase, simultaneously or immediately prior to the addition of antibody, combination is prevented until the substrate is exhausted. This is shown in Fig. 1 (insert, curve D) showing that inhibition starts slowly after substrate is utilized, as compared to the rapid inhibition in the absence of substrate. This "protection" (14) has also been shown in the case of other enzyme-antibody systems. However, once combined, antibody is not displaced by substrate, to any significant extent, as indicated by the fact that the Km of the partially inhibited enzyme equals that of the free enzyme (9).

Phosphate Effect on Deaminase-Antibody Combination. Phosphate is a competitive inhibitor of both chicken and rabbit deaminase (5, 12) and therefore "binds" at the same site as substrate. It should block combination with antibody in the same way as does AMP. However, it apparently increases the forward rate constant and also increases the absolute amount of inhibition, at equilibrium, that will be obtained with a given amount of antibody (9 and unpublished observation). Fig. 2 is a graph of the inverse of the deaminase activity *versus* time. The slope of these lines is proportional to the apparent rate constant of combination of the

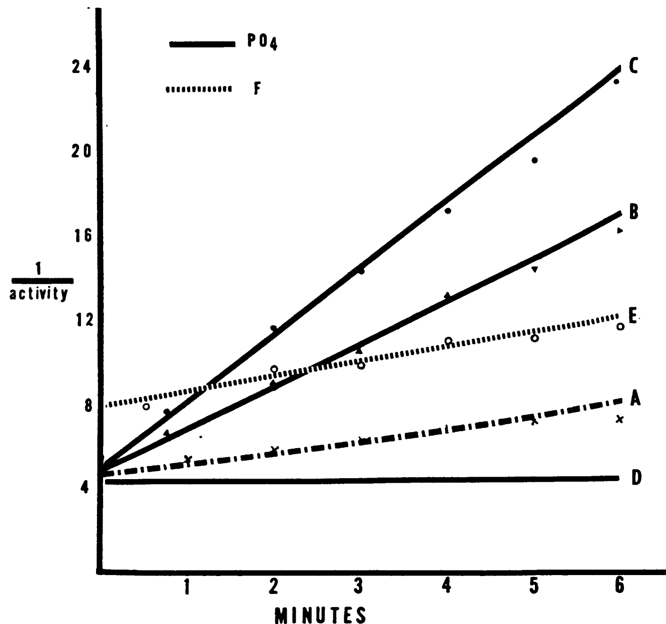

FIGURE 2 Effect of phosphate ion on the rate constant of deaminase-antibody combination. Conditions as described for the experiment shown in Fig. 1, insert, curve E. See text.

deaminase and its antibody, and inasmuch as the enzyme and antibody are equal in each of these experiments, the slopes are a measure of the rate of combination as well. Fig. 2, line A, shows the rate of enzyme inhibition by antideaminase in the absence of phosphate. Lines B and C show the increased rate constant in the presence of 0.1 and 0.2 M phosphate. Line D shows that in the presence of 0.2 M phosphate and control serum, under exactly the same conditions as in experiments B and C, the deaminase is not inhibited. The lack of inhibition by phosphate is not surprising because the concentration of phosphate is only 0.02 M after pipetting the enzyme-serum-PO_4 mixture into the substrate. This concentration of phosphate does not inhibit the enzyme in the presence of these substrate concentrations. Yet, as seen, with exactly the same concentration and under the same conditions, it does potentiate the enzyme inhibition by the antibody (curves B and C). Curve E shows that the rate constant of deaminase-antibody combination in the presence of 1×10^{-3} M fluoride is not changed, in spite of the fact that the enzyme is 50 per cent inhibited by this non-competitive inhibitor. One may suggest then that the competitive inhibitor allows more complete binding by the antibodies.

Creatine Kinase Inhibition. Creatine kinase requires three substrates (15), creatine hydrate, ATP, and Mg^{++} ion. It has been clearly shown by both groups (16, 17) working with creatine kinase that each of the substrates is bound to the enzyme. Equilibrium dialysis experiments showed that 2 moles of each of the substrates was bound per mole of protein with the exception of Mg^{++} of which 4

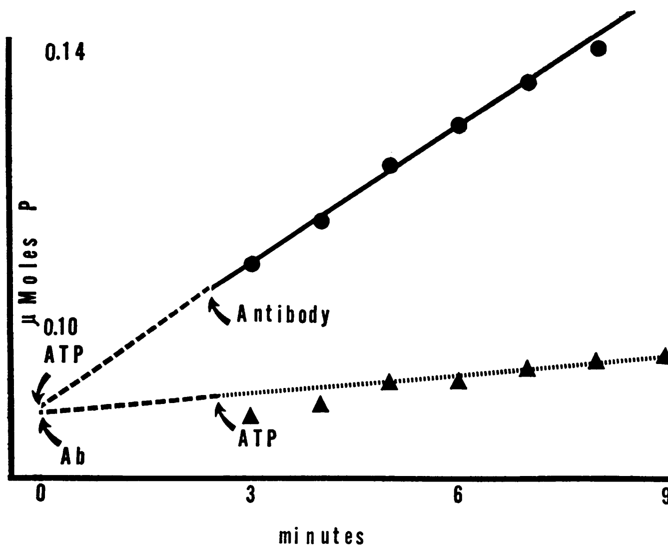

FIGURE 3 Substrate protection against antibody inhibition of creatine kinase is afforded only when all substrates are added before the addition of antibody. Substrate concentrations are similar to those of Fig. 1, main figure. The rate of creatine phosphate production (P) is a direct measure of the free enzyme concentration.

were bound. The creatine and creatine phosphate binding were independent of Mg concentration but the ATP and ADP binding was a function of the Mg^{++} concentration which indicated that some complex was formed between these two substrates in binding to the protein (17).

However, creatine kinase is *not* protected against combination with its antibody by the simultaneous or prior addition of any one or a combination of two of the substrates. Addition of the three substrates prior to the antibody does confer protection. This is illustrated in Fig. 3 which shows that creatine kinase incubated with two substrates (creatine + Mg^{++}) is inhibited upon the addition of antibody 2.5 min. prior to the addition of ATP (lower curve), while it is not inhibited as fast when the third substrate is added 2.5 min. prior to the antibody, *i.e.,* when the enzyme is already working. The same relations obtain when creatine is added last.

Obviously, as the affinity of the antibody is much higher than that of the substrate, unbound molecules of enzymes will be combined quite rapidly if the antibody concentration is too high.

DISCUSSION

The determination of the rate constant of combination of an enzyme and its antibody is based upon the assumption that the enzyme is *completely* and immediately inhibited upon combination with its specific antibody, before secondary complexes are formed. An equilibrium constant may also be determined by maintaining the antibody concentration constant in each tube and adding decreasing amounts of enzyme to it. The per cent inhibition of the added enzyme at equilibrium may then be converted into moles of bound enzyme and a plot of the ratio of bound/free enzyme *versus* moles of bound enzyme yields a straight line with a negative slope, which is equivalent to the equilibrium constant. This graph gives values of the order of 10^9 liter/mole for creatine kinase, but technical difficulties and occasional tendencies of this enzyme to increase activity on dilution, make the determination of an exact value difficult.

Very much in accord with the above findings on substrate protection of creatine kinase is the fact that much greater protection against heat denaturation has been observed with creatine kinase when all three substrates are present (15) than with any one singly. Previous work (14) has indicated that substrate protects enzyme against antibody combination, but at least one case, that of lactic acid dehydrogenase from rabbit muscle, has been shown to be an exception (18), for neither the oxidized nor reduced form of the two substrates added singly prevented combination. No kinetic studies of enzyme-antibody combination in the presence of both substrates were carried out in that work.

At first impression these results might be interpreted in terms of steric hindrance, for the binding of the antibody anywhere in the vicinity of the substrate site would

effectively block the approach of the substrate. Prior binding of the substrate could effectively prevent the binding of the antibody by a similar mechanism. However, the present results do not seem to fit this interpretation easily, for phosphate does not prevent binding of the deaminase antibody, although its kinetics as a competitive inhibitor indicate that it is bound at the same site on the enzyme as AMP which does protect against antibody inhibition. On the contrary, it seems to enhance the binding of more enzyme molecules by the antideaminase. However, its smaller size and charge-altering properties perhaps induce better fit with the antibodies after it is bound, rather than blocking as does the larger substrate. Furthermore, the mechanism of simple steric hindrance does not seem adequate in the creatine kinase–antibody system, for antibody combination is not sterically hindered by prior binding of two of the three components that form the substrate for this enzyme. The enzyme is protected from antibody combination when it is working. Because of the size of the antibody molecule, it seems unlikely that if it slips into position after the Mg-ATP is bound, it could also bind in the same position after the Mg and creatine are bound, for by the nature of the transfer reaction these substrates should be bound to the protein rather close to each other. If the idea of simple steric hindrance is to apply in this case, one might have to assume that the binding of the substrates experienced during equilibrium dialysis (16, 17) studies has no geometric relation to the binding that obtains during activation.

One interpretation of these results, especially those with creatine kinase, suggests that the configuration of a large enough part of the enzyme is altered during activation by substrates so that combination with antibody is no longer able to occur. Previous work has suggested that the antigen is deformed during combination with antibody (19). Recent work showing a change in optical rotation during the formation of "antigen-poor" complexes (20) supports this suggestion. Reviews of the enzyme literature (7), especially Koshland (in addition to his voluminous later work), Lumry, Linderstrom-Lang, and Schellman, and more recently, Blum (21) and others, also suggest, on the basis of kinetic and thermodynamic arguments, a lability of enzyme structure or at least the active site. Changes in the optical rotation of ribonuclease in the presence of pyrophosphate (22), and in glyceraldehyde-3-phosphate dehydrogenase upon DPN binding (23, 24) as well as immunochemical studies on urea-treated penicillinase (25) also indicate a conformation change during substrate binding.

The optical rotation and rotatory dispersion of creatine kinase are being studied here, and evidence recently reported (26) shows a small change in optical rotation of the enzyme only when the enzyme is working. This strongly supports the most probable interpretation of the immunoenzymological results reported here, that of a conformation change. However, even this evidence is not irrefutable, as the formation of a new assymetric center, upon the binding of small molecules to proteins, is possible (27).

If a functionally related conformation change does indeed occur, it might be viewed as an "induced fit" (see Koshland in reference 7), which as a mechanism for general enhancement of specificity must be evaluated, or at the other extreme, as a physiologic process, like a stimulus response, in which information is transmitted to the protein causing a specific conformational change. If individual conformation changes of proteins occur during embryogenesis, aging, denervation atrophy, and during various disease states, we begin to see a role that the complex structure of proteins may play that is suggestive of the biological sensitivity to environment seen in organisms at higher levels of organization.

This work was supported by the National Science Foundation under Grant G5715.
Received for publication, February 29, 1961.

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