Two Antigenic Sites of Tissue Transglutaminase†

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ABSTRACT: The immunization of rabbits with purified guinea pig liver transglutaminase resulted in the appearance of two antibody populations against the enzyme: one which reacted only with the Ca^{2+}-enzyme complex and another which reacted with the intact as well as the Ca^{2+}-enzyme. The Ca^{2+}-induced conformational change of the enzyme molecule exposes a new antigenic determinant which initiates the production of a specific antibody population. When the glutamine substrate of the enzyme was a dipeptide, the result of the interaction of the Ca^{2+}-enzyme and its isolated specific antibody was an apparent activation of the catalytic activity. However, when protein substrates were used, an inhibition was observed. The characterization of the mechanism of the activation and the inhibition has led to the conclusion that the consequence of the interaction of the Ca^{2+}-enzyme and its specific antibody is not only a limited steric hindrance of the active center but, besides that, a stabilization of the otherwise labile Ca^{2+}-enzyme. The other antibody population reacts with both forms of the enzyme and its inhibitory effect, which has been observed in each assay, could be due to a prevention of the Ca^{2+}-induced formation of the active enzyme.

Studying enzymes by the use of their specific antibodies has proved to be a fruitful approach on several occasions. The interaction between enzymes and their specific antibodies leads generally to a reduction in the enzyme activity, although it has also been reported that sometimes antibodies can stimulate or stabilize the activity of the enzyme (reviewed by Arnon, 1974). Besides the usefulness of antibody in functional studies of enzymes, it is also an excellent tool for the detection of structural characteristics and conformational changes of various enzymes (Arnon, 1974).

Tissue transglutaminase (molecular weight: 85 000), an enzyme which is widely distributed in various tissues and organs (Chung, 1972, 1975), catalyzes an acyl transfer reaction at the carboxamide groups of protein-bound glutamine with a variety of compounds containing a primary amino group. The reaction yields new γ-amide bonds and the replaced amide appears as ammonia. In the absence of an exogenous amine, ϵ-amino groups of appropriate protein-bound lysyl residues can serve as substrates and intra- or intermolecular ϵ (γ-glutamyl) lysyl cross-links are formed. The physical, chemical, and catalytic properties of the purified enzyme have been studied in detail, including the active site mapping (reviewed by Folk and Chung, 1973; Folk and Finlayson, 1977). The enzyme requires Ca^{2+} ions which induce conformational alterations essential for the catalytic activity. In spite of these studies, the biological role of the enzyme is not known.

Since a specific inhibitor of the tissue transglutaminase which could be used for structural as well as biological studies has not yet been found, the production of specific antibodies against the enzyme seemed desirable. The characterization of the antibody and its interaction with the enzyme could lead to an antibody preparation suited for in vivo studies and, in addition, the study of the enzyme–antibody interaction may supply further information about the conformational state of the enzyme.

Materials and Methods

Benzyloxycarbonyl-L-glutaminylglycine (*Z*-Gln-Gly)[1] and purified β-casein A were the kind gifts of Dr. J. E. Folk; [^{14}C]methylamine hydrochloride, carrier-free ^{125}I, and the Bolton–Hunter reagent were purchased from New England Nuclear; Sepharose 4B and 6B were from Pharmacia; goat anti-rabbit IgG was from Cappel Laboratories. All other reagents were either reagent grade or the best available.

Enzymic Assays. Highly purified guinea pig liver transglutaminase was a generous gift of Dr. S. Chung (specific activity, 12.4 determined by the hydroxamate assay). This will be called the *intact enzyme* to distinguish from the *Ca-activated enzyme.*

Assay I. Measurement of the transglutaminase-catalyzed incorporation of [^{14}C]methylamine into the synthetic dipeptide *Z*-Gln-Gly was carried out as follows: 10 μL of Tris–acetate buffer (1.0 M, pH 7.5), 1 μL of dithiothreitol (0.1 M), and 1 μL of EDTA (0.1 M) were mixed (buffer mixture) and combined with 25 μL of 0.2 M *Z*-Gln-Gly, 25 μL of the enzyme solution (diluted in 0.15 M NaCl–5 mM citrate, pH 7.5), and 25 μL of another buffer solution (0.15 M NaCl/10 mM Tris–acetate, pH 7.5) which contained different amounts of rabbit IgG antibodies. The catalytic reaction started 15 min later on the addition of 10 μL of [^{14}C]methylamine (50 mM) and 10 μL of 0.3 M $CaCl_2$. In one part of the experiments, when the enzyme was preincubated in the presence of 30 mM $CaCl_2$ before its addition to the reaction mixture, the reaction started by the addition of methylamine only. After 30 min incubation (if not otherwise indicated), the reaction was stopped by the addition of 10 μL of 0.1 N HCl. The arrested reaction mixture was then applied onto a 2.0 × 0.1 cm column of Dowex 50W-X8 in the hydrogen cycle. The reaction product of *Z*-Gln-Gly and [^{14}C]methylamine was washed off the column five times with 1.0 mL of H_2O into counting vials and their radioactivity was determined using Aquasol scintillation fluid.

Assay II. In the case of the measurement of the transglutaminase-catalyzed incorporation of [^{14}C]methylamine into

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[1] Abbreviations used: *Z*-Gln-Gly, benzyloxycarbonyl-L-glutaminylglycine; IgG, immune globulin γ chain; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

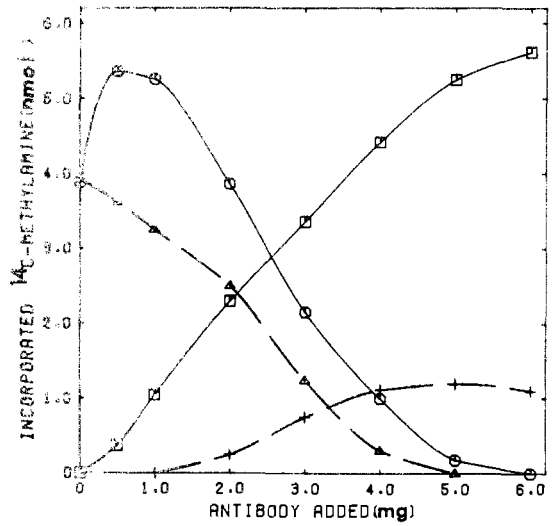

FIGURE 1: Immunoprecipitation of tissue transglutaminase with the IgG fraction of anti-transglutaminase antiserum. For the immunoprecipitation, aliquots of purified transglutaminase (30 μg in 0.15 M NaCl–5 mM citrate (pH 7.5)) were reacted with varying amounts of the IgG fraction at 4 °C overnight (Δ and +). Accordingly, the precipitation was carried out after preincubation of the enzyme in the presence of 30 mM $CaCl_2$ (□ and ○). The precipitates were centrifuged at 3000 rpm for 3 min; the supernates were separated and the immunoprecipitates were resuspended in the same buffer using a glass rod. Transglutaminase activity was assayed by Assay I using 50-μL aliquots of both fractions [(Δ and ○) activities in the supernates; (+ and □) activities in the precipitates]. Enzyme activity is expressed in nmol of incorporated [^{14}C]methylamine.

β-casein, the reaction mixture and the procedure were essentially the same as for the *Z*-Gln-Gly substrate, except for the addition of 25 μL of 4% casein instead of the *Z*-Gln-Gly and the technique used for the separation of casein from the reaction mixture. The reaction mixture was pipetted onto strips of DEAE-cellulose precoated plastic sheets after stopping the reaction, and, following a single paper chromatography, the radioactivity of the casein containing part of the strips was measured.

The determination of the extent of the transglutaminase-catalyzed cross-linking of ^{125}I-labeled fibrinogen, as well as the coupling of it to the cell surface, was carried out as we previously described (Fésüs and Laki, 1976).

The K_m and K_i values were calculated according to Dixon and Webb (1964).

Immunological Methods. Anti-guinea pig liver transglutaminase antibodies were produced in New Zealand white rabbits. Two milligrams of the purified intact enzyme was injected subcutaneously into the animals at weekly intervals, three times in complete, then in incomplete Freund adjuvant. Blood was collected 5 weeks after the first injection; the pooled serum was precipitated in the presence of 33% ammonium sulfate, and the IgG fraction was separated by gel filtration on a 6% agarose column equilibrated by a citrate buffer (0.5 M citrate (pH 7.6)–0.075 M NaCl–0.02% sodium azide). The IgG fraction was concentrated and dialyzed against 10 mM Tris–acetate buffer (pH 7.5) containing 0.15 M NaCl, and the protein concentration was determined spectrophotometrically assuming a specific extinction coefficient of $E_{1cm}^{1\%} = 14.0$ at 280 nm (Porter, 1957). A single line of precipitation was obtained by testing the antibodies against the purified transglutaminase on the Ouchterlony immunodiffusion plate.

Purified antibodies were obtained by adsorption of the IgG fraction to the purified transglutaminase, coupled covalently to cyanogen bromide activated Sepharose 4B beads (Porath et al., 1967), and elution with 0.1 M sodium citrate at pH 2.2. The eluted peak was titrated to pH 7.0 with 1 M NaOH, dialyzed against 10 mM Tris–acetate, pH 7.5, containing 0.15 M NaCl, and the protein concentration was determined spectrophotometrically. The monovalent antibody fragments (Fab) were also separated by the use of this technique following a papain digestion of the purified antibodies (Porter, 1959).

Quantitative precipitin tests were performed with the purified antibody. The enzyme was labeled by the Bolton–Hunter reagent (Bolton and Hunter, 1973). The specific activity was 0.8×10^{-2} μCi per μg of protein calculated from the adsorbance of the labeled enzyme assuming $E_{1cm}^{1\%} = 15.8$ at 280 nm (Folk and Cole, 1966). Increasing amounts of the labeled enzyme were added to a constant amount of the antibody. The precipitates formed overnight at 4 °C were washed and the amount of antigen in the precipitate was determined by measuring its radioactivity in a γ counter. The antigen–antibody binding ratios were estimated according to Farr (1958) in duplicate precipitin experiments in which goat anti-rabbit IgG was added to the reaction mixture following the enzyme–antibody interaction.

Results

The IgG fraction of the transglutaminase antiserum precipitated the enzyme irrespective of whether it was the intact one or the Ca^{2+}–enzyme (Figure 1). When the antibodies reacted with the intact enzyme, the catalytic activity, using Assay I for its measurement, was inhibited in the supernate at high antigen excess as well as in the precipitates at higher antibody concentrations, but the inactivation was never complete. On the other hand, when the Ca^{2+}–enzyme was the antigen, the catalytic activity increased at high antigen excess in the supernate. The total enzyme activity of the supernate and the precipitate at higher antibody concentrations was always above the activity in the control tube not containing antibody.

Purified antibody was prepared from the IgG fraction of the anti-transglutaminase antiserum by affinity chromatography. The effect of this purified antibody preparation was tested in Assay I. The order of the addition of the reactants relating to the addition of the antibodies was varied. An inhibition curve, reaching approximately 75% inhibition of the original enzyme activity, was obtained when the addition of Ca^{2+} into the mixture followed the antibody addition (Figure 2). However, when the same antibody preparation reacted with the Ca^{2+}–enzyme, an activation reaching about 125% of the original activity could be obtained (Figure 2). In the control experiments, the presence of the same amount of nonspecific rabbit IgG, prepared in the same way as the anti-transglutaminase IgG, did not influence the enzyme activities in either case. When the same experiments were repeated, using the monovalent Fab fragment of the purified antibodies instead of the whole molecule, similar inhibition and activation curves were observed (not included in figures) showing that the formation of enzyme–antibody precipitate does not affect either the activation or the inhibition phenomenon. There was no difference in the results when the order in which the substrates were added to the intact or the Ca^{2+}–enzyme was varied relating to the addition of the purified antibodies.

In order to clarify whether this double effect of the antibody on the enzyme activity is the consequence of the presence of two types of antibody in the preparation or only one type which affects the two forms of the enzyme in a different manner, quantitative precipitin tests were performed using ^{125}I-labeled enzyme in its intact or Ca^{2+}–enzyme form as antigens. As shown in Figure 3, the maximal amount of the intact enzyme precipitated in the equivalence zone was approximately 40%

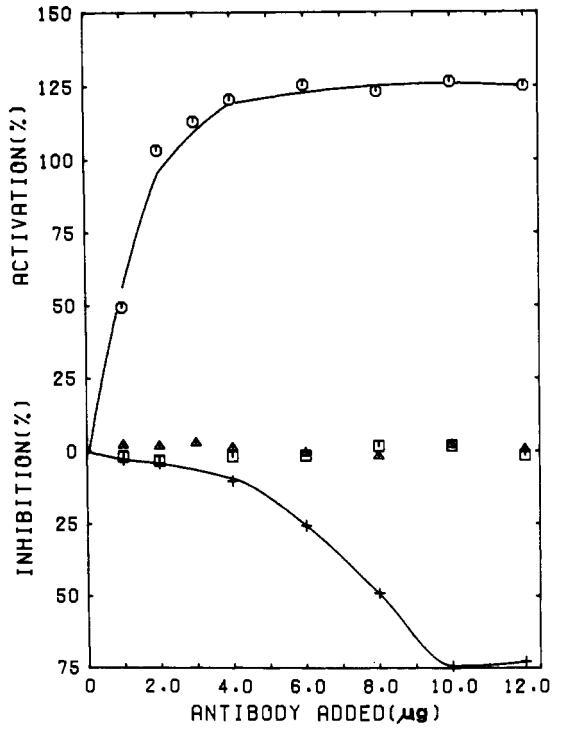

FIGURE 2: The effect of various amounts of the purified antibody preparation on the activity of 1.0 μg of intact (+) or Ca^{2+}-enzyme (O). In the control experiments, normal IgG was added to the intact (Δ) or the Ca^{2+}-enzyme (□). Enzyme activity was determined by the *Z*-Gln-Gly assay.

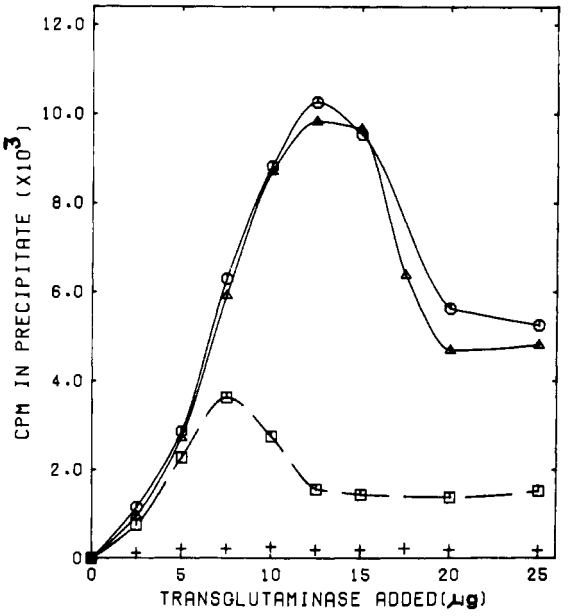

FIGURE 3: Quantitative precipitin test of ^{125}I-labeled transglutaminase using two different antibody preparations. Purified antitransglutaminase antibodies [(□- -□) intact enzyme; (O—O) Ca^{2+}-enzyme]; the same experiment with the antibody fraction that was not precipitated with the intact enzyme and separated by a second affinity chromatography step [(+) intact enzyme; (Δ—Δ) Ca^{2+}-enzyme]. The IgG concentration is 30 μg in each tube.

of that precipitated when the Ca^{2+} form of the labeled enzyme was used. As such differences may be the consequence of a change in the ratio of soluble to precipitating complexes, these experiments were repeated with an excess of goat anti-rabbit IgG added to each tube after 15 min preincubation of the anti-transglutaminase antibody-enzyme (intact or the Ca^{2+}-enzyme) mixture. The same difference in the antibody-antigen

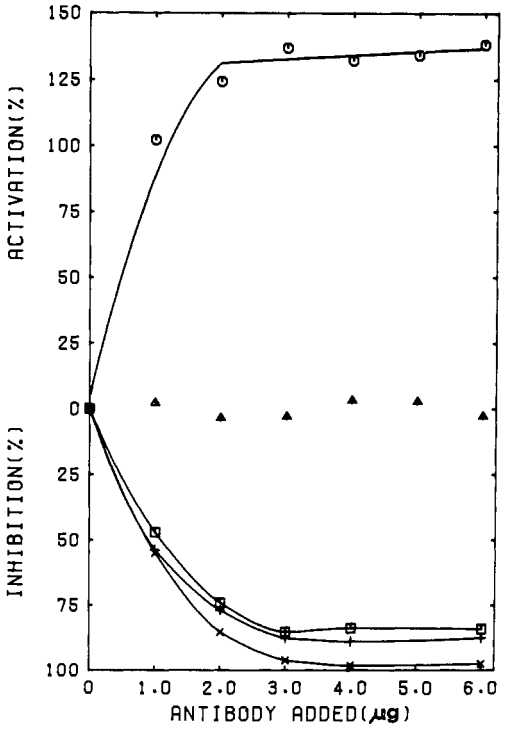

FIGURE 4: Effect of the antibody population that is not precipitated with the intact enzyme on the Ca^{2+}-enzyme (1 μg of transglutaminase in each case, preincubated in the presence of 30 mM $CaCl_2$ before addition of the antibody) tested by Assay I. (O—O) Assay II; (□—□) the assay measuring the cross-linking of bovine fibrinogen by the enzyme (+—+); the technique detecting the coupling of fibrinogen to the surface proteins of cells by the enzyme (X—X). Δ represents the results obtained when nonspecific rabbit IgG was used in the membrane-coupling assay.

ratio was obtained as in the simple quantitative test (not included in the figure). To determine whether the antibody fraction that is not precipitated with the intact enzyme is a selected population or not, in a separate experiment the purified antibody preparation was precipitated by the intact enzyme in the equivalence zone. The supernate was applied onto the Sepharose 4B column, having the transglutaminase immobilized to its beads, then the adsorbed antibodies were eluted, concentrated, dialyzed, and examined for its precipitation behavior and interaction with the enzyme in various assay systems. As seen in Figure 3, this antibody population (adding the same amount of IgG as previously) was not precipitated when the intact enzyme was used as antigen, but gave a precipitin with the Ca^{2+}-enzyme. The molar ratio of the antibody to the Ca^{2+}-enzyme at the equivalence zone was approximately the same as it was when the same amount of the total antibody population was studied. When a similar attempt as above was made to separate an antibody population which was not precipitated with the Ca^{2+}-enzyme, we failed to find antibody in the supernate of the precipitin formed in the presence of the Ca^{2+}-enzyme and the whole antibody population. The last two observations clearly show that the antibody precipitated with the intact enzyme can react with and precipitate the Ca^{2+}-enzyme as well.

The antibody population that is not precipitated with the intact enzyme was tested in various assays of tissue transglutaminase. As shown in Figure 4, the addition of increasing amount of this antibody to the Ca^{2+}-enzyme resulted in its activation when tested in Assay I. On the other hand, this antibody population inhibited the enzyme activity very effectively in assays when the glutamine substrate was a protein (casein-methylamine assay, cross-linking of fibrinogen, cou-

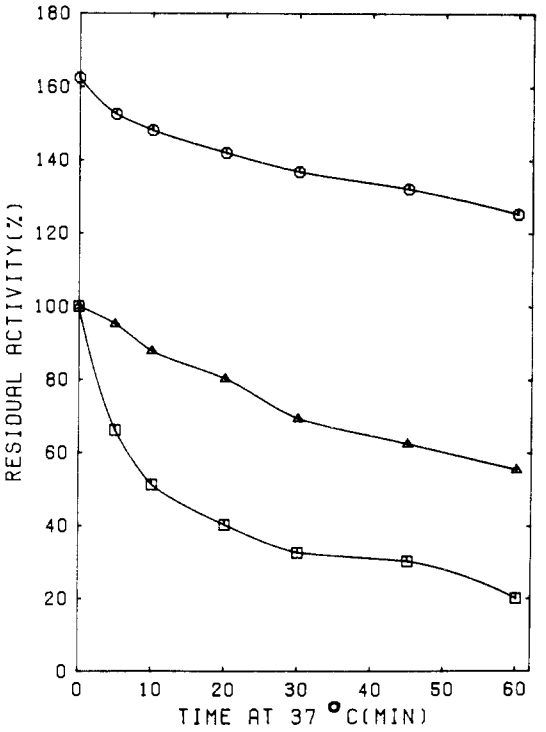

FIGURE 5: Residual activities of the intact and the Ca^{2+}-enzyme after incubation at 37 °C for various periods of time in the presence of nonspecific IgG or the specific antibody population that is not precipitated with the intact enzyme; (□—□) Ca^{2+}-enzyme in the presence of nonspecific IgG; (Δ—Δ) intact enzyme in the presence of nonspecific IgG; (○—○) Ca^{2+}-enzyme in the presence of the specific antibody population. Experimental conditions: 10 μg of enzyme and 15 μg of IgG were incubated at 37 °C in 1 mL of the buffer mixture (see Materials and Methods) in the presence of 50 mM *Z*-Gln-Gly and 30 mM Ca^{2+} (the latter was omitted in case of the intact enzyme). At various intervals after starting the incubation, 100-μL aliquots were removed and the enzyme activity was determined after the addition of methylamine (and Ca^{2+} in case of the intact enzyme) and incubation of the samples for an additional 15 min.

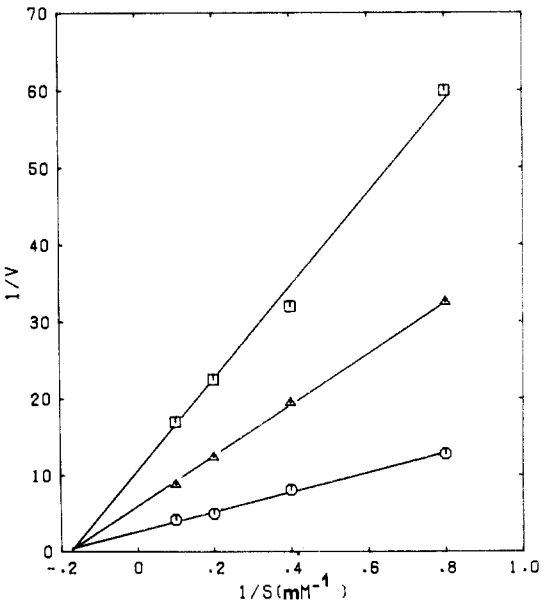

FIGURE 6: Effect of the antibody that is not precipitated with the intact enzyme on incorporation of methylamine into β-casein by the enzyme. (○—○) No antibody; (Δ—Δ) 1 μg of antibody; (□—□) 3 μg of antibody. Plot of reciprocal velocities (nmol of [^{14}C]methylamine incorporated into β-casein per min per 1 μg of enzyme) against substrate concentration.

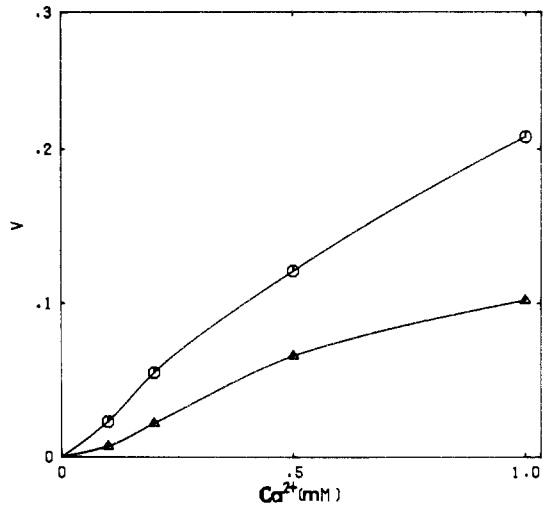

FIGURE 7: Effect of various Ca^{2+} concentrations on the velocity of transglutaminase catalyzed methylamine incorporation into β-casein. The Ca^{2+} was added after 15-min preincubation of 1 μg of the enzyme with 15 μg of nonspecific IgG (○—○) or specific, purified antibody (Δ—Δ) in the presence of the buffer mixture and casein. Velocities are given in nmol of [^{14}C]methylamine incorporated per min per 1 μg of enzyme.

pling of fibrinogen to the cell surface). When the Ca^{2+}-enzyme was preincubated with the casein substrate before the addition of the antibody, the same inhibition could be observed (not included in the figure).

The mechanism of the activation observed with Assay I was further studied by incubating the Ca^{2+}-enzyme with either nonspecific rabbit IgG or its specific antibody at 37 °C for various periods of time in the assay mixture before starting the reaction by the addition of methylamine. The Ca^{2+}-enzyme was found very labile, losing 50% of its original activity within 10 min and about 80% within 1 h (Figure 5). The stability of the intact enzyme was much higher. The presence of the specific antibody protected the Ca^{2+}-enzyme from the rapid inactivation.

In order to get more information about the interaction of the Ca^{2+}-enzyme with its specific antibody, the effect of two concentrations of the antibody on the enzyme was determined at various substrate concentrations in the casein assay. As seen in Figure 6, the inhibition was noncompetitive in both cases with a K_m value of 5.2×10^{-3} M and K_i of 1.6×10^{-7} M.

The whole population of the purified antibody preparation was used to study the result of the interaction of the intact enzyme and the antibody which reacts with this form of the enzyme. In order to prevent the interference of the other antibody as much as possible, excess amount of the purified antibody preparation was added to the intact enzyme, presumably enough for the interaction with the whole quantity of the enzyme present, and the catalytic reaction was started after 15 min of preincubation. The result was always an inhibition of the catalytic reaction in all the assay systems. (Inhibitions in per cent of the original activity testing the effect of 15 μg of the purified antibody on the activity of 1 μg enzyme: 76% in Assay I, 79% in Assay II, 81% in the case of fibrinogen cross-linking, and 83% when the coupling of fibrinogen to the cell surface was studied.) In another experiment, the effect of various concentrations of Ca^{2+} on the enzyme activity was determined in Assay II after preincubation of the enzyme with normal IgG or the purified antibody preparation. As shown in Figure 7, the higher the Ca^{2+} concentration, the more effective inhibition could be obtained; that is, the effect of the same amount of antibody was more pronounced when higher Ca^{2+} concentrations were used.

Discussion

The antibody population which we produced against the guinea pig liver transglutaminase in rabbits was not homogeneous. It was observed that, depending upon whether the intact enzyme or the one previously submitted to the Ca^{2+}-induced conformational change was used as antigen, the result of the enzyme–antibody interaction was different. When the IgG fraction of the anti-transglutaminase antiserum was used for the studies, the Ca^{2+}-enzyme was precipitated at a lower antibody concentration than the intact enzyme and the total activity in the tubes, estimated by Assay I, was always higher than in the controls which did not contain anti-enzyme antibody. On the contrary, the intact enzyme was always inhibited in these experiments. When the specific anti-transglutaminase antibodies were purified from the IgG fraction by adsorption to immobilized transglutaminase, this antibody preparation had similar properties; i.e., the addition of increasing amounts of antibodies to the Ca^{2+}-enzyme resulted in increasing activation, but an inhibition when they reacted with the intact enzyme. The results of the quantitative precipitin test showed that the existence of two populations of antibodies was responsible for these results: one that did not precipitate with the intact enzyme but did with the Ca^{2+}-enzyme, and another which was precipitated by both. When we repeated the adsorption of the antibodies with the amount of the IgG fraction as earlier using the same column but equilibrated previously by a Ca^{2+}-containing buffer, the elution profile was not different either qualitatively or quantitatively (not included in the results). Furthermore, the antibody population, which was not precipitated in the presence of the intact enzyme, could be absorbed to the immobilized intact enzyme.

Our experiments suggest that the immobilized enzyme for the antibody may look like the Ca^{2+}-enzyme without actually being the active enzyme. Nevertheless, we do not have to propose the change occurring during immobilization to be similar to that occurring during the Ca^{2+} activation. The change, in fact, is not similar because the enzyme cannot be made active (a sign of change) but this does not exclude the appearance of an antigenic site during immobilization identical with that appearing during Ca^{2+} activation.

The separation of the antibody population that was not precipitated with the intact enzyme was accomplished and the mode of its interaction with the Ca^{2+}-enzyme was characterized. As this antibody population reacts only with the Ca^{2+}-enzyme, it is obvious that the Ca^{2+}-induced conformational change of the enzyme molecule exposes a new antigenic site. Apparently when the antibody interacts with this region, the catalytic activity of the enzyme depends upon the substrate which is used to test it. When the glutamine substrate is a small molecule, such as the dipeptide, *Z*-Gln-Gly, the result of the enzyme–antibody reaction is activation; when it is a protein, such as casein, fibrinogen, or membrane proteins, an inhibition occurs.

The antibody population which reacts with both forms of the enzyme has not been isolated. Its effect on the intact enzyme was studied by assuming that, when the intact enzyme was preincubated in the presence of an excess amount of the whole antibody population, the enzyme reacted with this particular population of the antibodies exclusively and there were only limited new enzyme–antibody interactions following the addition of Ca^{2+} at the starting point of the reaction. The result was inhibition, though never complete, of the catalytic activity in each of the assays.

An attempt was made to find out the mechanism of the activation of the Ca^{2+}-enzyme by its specific antibody. There are examples when the enzyme–antibody interaction results in an increase in the enzyme activity. Such an effect was manifested when enzymes reacted under nonoptimal conditions (Messer and Melchers, 1969; Lehman, 1970), or when the antibody stabilized the naturally labile form of an enzyme affecting its conformational structure (Suzuki et al., 1969; Arnon, 1974). In our experiments, the Ca^{2+}-enzyme was found to be very labile at 37 °C in the presence of the *Z*-Gln-Gly substrate, (the same situation occurs probably during the catalytic reaction too), and the presence of the antibody could stabilize the labile Ca^{2+}-enzyme, thereby resulting in an apparent activation.

Inhibition of enzyme activity after interaction with its antibody may be explained in general by conformational changes of the enzyme and/or steric hindrance of the catalytic site (Arnon, 1974). Conformational distortion at the substrate binding site of the enzyme has been found with several enzymes (Samuels, 1961, 1963; Arnon, 1974). In these cases, the enzyme was protected from the inhibition by preincubation with substrate. In our experiments, the antibody (that does not precipitate with the intact enzyme) inhibited the Ca^{2+}-enzyme in a noncompetitive manner, thus excluding the possibility that it attaches to the substrate binding site exclusively. On the basis of our experiments, the possible explanation of the inhibition by this antibody is the following: the binding of the antibody to the antigenic determinants exposed by the Ca^{2+}-induced conformational change results in steric hindrance of the active site as far as the catalytic activity toward protein substrates is concerned, but the extent of the steric hindrance is not enough to inhibit the catalytic reaction with the small substrate, *Z*-Gln-Gly. This phenomenon is similar to those which have been demonstrated for several other enzymes when the extent of inhibition by antibodies could be related to the size of the substrate (Branster and Cinader, 1961; Fazekas de St. Groth, 1963; Arnon and Schechter, 1966; Shapira and Arnon, 1967)—the larger the substrate, the higher the extent of inhibition.

The mechanism of the inhibition of the intact enzyme by the antibody population which reacts with both forms of the enzyme seems to be different. First of all, it is highly unlikely that its interaction with the Ca^{2+}-enzyme inhibits the enzyme activity, because there is only a slight difference in extent of the activation of the Ca^{2+}-enzyme when one compares the effect of the total antibody population (Figure 2) and the isolated specific antibody of the Ca^{2+}-enzyme (Figure 4). Therefore, its inhibitory effect is very likely due to the inhibition of the formation of the active enzyme. The direct relationship between the Ca^{2+} concentration and the extent of the inhibition further supports this explanation; i.e., the inactive (intact) conformational structure of the enzyme is "frozen" by this antibody preventing the Ca^{2+}-induced activation.

The most probable explanation of the production of the two types of the antibodies is that the subcutaneous injection of the enzyme is followed by the Ca^{2+}-induced conformational change exposing new antigenic determinants and inducing the production of another type of antibody in the rabbits. As both forms of the transglutaminase (the intact or the Ca^{2+}-enzyme) may exist under in vivo conditions, and considering the wide range of the potential substrates of this enzyme, these antibody preparations may be used in studies to clarify its biological role.

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Spontaneous, Reversible Protein Cross-Linking in the Human Erythrocyte Membrane. Temperature and pH Dependence†

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ABSTRACT: Changes in pH significantly affect the morphology and physical properties of red cell membranes. We have explored the molecular basis for these phenomena by characterizing the pattern of protein disulfide cross-linkages formed spontaneously in ghosts exposed to acid pH or elevated temperature (37 °C). Protein aggregation was analyzed by two-dimensional polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Incubation of ghosts at pH 4.0 to 5.5 (0–4 °C) yielded (i) complexes of spectrin and band 3, (ii) complexes of actin and band 3, (iii) band 3 complexes, i.e. dimer and trimer, and (iv) heterogeneous aggregates involving spectrin, band 3, band 4.2, and actin in varying proportions. Aggregation was maximal near the isoelectric points of the major membrane proteins, and appeared to reflect (i) the aggregation of intramembrane particles including band 3 and (ii) more intimate contact between spectrin–actin meshwork and band 3.

Changes in pH significantly affect the morphology and physical properties of the human erythrocyte membrane. Electron microscopic studies on intact red cells and isolated ghosts revealed several reversible pH-dependent phenomena such as changes in shape (Weed and Chailley, 1973; Nicolson, 1973), aggregation of intramembrane or freeze-etch particles (Pinto da Silva, 1972; Elgsaeter and Branton, 1974), clustering of surface anionic sites composed of *N*-acetylneuraminic acid residues (Nicolson, 1973), and clustering of ferritin binding sites (Pinto da Silva et al., 1973). It has been suggested (Elgsaeter et al., 1976) that these rearrangements reflect the isoelectric precipitation of spectrin and actin, the peripheral proteins which form a meshwork on the membrane–cytosol interface (Kirkpatrick, 1976), and are mediated through their interaction with transmembrane proteins.

To elucidate the molecular basis of such interactions, we have investigated spontaneous cross-linking of membrane proteins produced by exposing ghosts to reduced pH. The composition of the cross-linked products has been analyzed by two-dimensional sodium dodecyl sulfate gel electrophoresis, in which resolution in the second dimension is preceded by dithiothreitol reduction to free individual polypeptides from complexes.

The data demonstrate maximal cross-linking in the pH range 4.5–5.0 and provide direct evidence of covalent bond formation between spectrin and the major transmembrane protein, band 3. The correlation between the formation of homologous band 3 complexes and the intramembrane particle aggregation is also discussed. A preliminary report of this study has been published (Liu et al., 1976).

Materials and Methods

Preparation of Erythrocyte Ghosts. Venous blood was collected from healthy volunteers into citrate–phosphate–dextrose anticoagulant and used within 2 weeks of storage at 4 °C. Red cells were isolated by centrifugation at 1000*g* for 15 min. The supernatant and buffy coat were discarded by aspiration, and red cells were washed three times with 3 vol-

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