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GLUTAMATE DEHYDROGENASE FROM HUMAN LIVER

III. ANTIBODY-BINDING SITES AND PROPERTIES OF THE ANTIGEN–ANTIBODY COMPLEX

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SUMMARY

Rabbit antibodies to human glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) were directed against those protein surfaces of the monomeric enzyme protein which were hidden inside the polymeric enzyme complex by juxtaposition of monomers. Antibodies precipitated only the monomers quantitatively. With increasing polymerization the number of free antibody-binding sites decreased. When immunological methods were employed, this change in the protein surface of human glutamate dehydrogenase during polymerization led to several enzymatically active precipitates in spite of the presence of only one protein and one antibody population. Therefore, atypical Heidelberger curves were found with increasing antigen concentration. When the precipitates were measured with increasing antibody concentration, a typical curve with resolution of the antigen–antibody complex in the presence of antibody excess was found only in the concentrations in which the antigen appeared mainly in the monomeric form. The possibility of the existence of soluble antigen–antibody complexes was eliminated by studying the supernatants of the Heidelberger curves in molecular sieve electrophoresis and gel chromatography on Sepharose 6B. The homologous and heterologous antibodies activated the human enzyme in the reaction with 2-oxoglutarate as substrate by 230% and the bovine enzyme by 380% of the original activity. In the reaction with glutamate as substrate, both enzymes were inhibited almost completely by antibodies to bovine glutamate dehydrogenase. The inhibition by antibodies to human glutamate dehydrogenase was in the range of 10%. It seems that simultaneous activation and inhibition within one immunological enzyme–anti-enzyme system was mediated by binding of antibodies at or near the allosteric sites with consequent change of the allosteric configuration. Antibodies and allosteric effectors such as ADP and GTP probably competed at these sites.

INTRODUCTION

The biochemical properties of crystalline glutamate dehydrogenase from human liver (L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) are very similar to those of all known animal glutamate dehydrogenases[1], particularly crystalline glutamate dehydrogenase from bovine liver. However, coenzyme analogues demonstrated that the steric configurations of the active centers are different[2]. Antibodies to crystalline human and bovine glutamate dehydrogenases in the heterologous systems showed the same to be true for the antigenic determinants[3]. Therefore, we performed further immunological investigations to determine the antibody-binding sites on the surface of human glutamate dehydrogenase and to compare our results with hitherto known biological data concerning this enzyme. We attempted to discover whether the change in the conformation of the human glutamate dehydrogenase surface due to various degrees of association and dissociation or due to different allosteric states induces changes in the available free antibody-binding sites. Furthermore we studied the antigenic determinants on the surface of the enzyme in order to provide an explanation of the previously observed stimulation of the enzymatic activity by the antibody[2].

In quantitative precipitation curves TALAL AND TOMKINS[4] found no linear correlation between glutamate dehydrogenase from bovine liver and its homologous antibodies, and FAHIEN *et al.*[5] found none between glutamate dehydrogenase from frog liver and the heterologous antibodies to bovine glutamate dehydrogenase; no explanation was offered for this phenomenon. Since preliminary observations showed a decrease of the antigen–antibody complex with rising enzyme concentration between crystalline human glutamate dehydrogenase and its homologous or heterologous antibodies (to bovine glutamate dehydrogenase), the human glutamate dehydrogenase–anti-human glutamate dehydrogenase system was studied to find the reasons for this atypical behaviour.

MATERIAL AND METHODS

Material

2-Oxoglutarate, NAD, NADH, ADP and GTP were purchased from Boehringer, Tutzing; L-glutamate and nitrobluetetrazoliumchloride, from Sigma Chem., St. Louis', phenazine methosulfate, from Serva-Entwicklungslabor, Heidelberg; polyvinylpyrrolidone (K30 pharm. purum, mol. wt. approx. 40 000) and tri-ethanolamine, from Fluka, Buchs; complete Freund's adjuvant, from Biological Laboratories, Detroit; hydrolyzed starch, from Connaught, Toronto; Sephadex G-200, Sephadex G-25 and Sepharose 6B, from Pharmacia, Uppsala; agar-Noble, from Difco, Detroit; agarose, from Behring, Marburg; and all other chemicals from Merck, Darmstadt.

Antigens

Human glutamate dehydrogenase was prepared as described by LEHMANN AND PFLEIDERER[1]. The specific activity of the crystalline preparations ranged from 74.4 to 102 units/mg with 2-oxoglutarate as substrate. The preparations were pure in high-voltage starch gel electrophoresis, gel filtration on Sephadex G-200 and

immunoelectrophoresis. Glutamate dehydrogenase of bovine liver was obtained from Boehringer, Tutzing.

Antiserums

After preliminary experiments, 12 mongrel rabbits were immunized with human glutamate dehydrogenase, and 12 with bovine glutamate dehydrogenase as follows: glutamate dehydrogenase was dialyzed against 0.2 M Sørensen buffer containing $1 \cdot 10^{-3}$ M EDTA, pH 7.5; denatured protein was removed by centrifugation at 36 000 × *g*; a 10-mg/ml solution was prepared by dilution with buffer and emulsified (1:1) with complete Freund's adjuvant by ultrasonics (Sonifier of Branson Co., Danbury). Rabbits were injected with 5-mg/ml solutions or emulsions. 10 mg of enzyme in complete Freund's adjuvant were injected in 5 equal portions of 0.4 ml emulsion (= 2 mg) into the 4 foot pads and intramuscularly. 28 days later, 5 mg glutamate dehydrogenase in complete Freund's adjuvant were injected (4 mg subcutaneously in the nuchal region and 1 mg intramuscularly). 7 days later the antibody content of serum was determined by nephelometry; when a high antibody titer was found, 60–80 ml blood was taken from the auricular artery and the animal was bled to death through the carotid artery under Nembutal anesthesia. We collected 100–150 ml of serum from each animal. When the antibody titer was low, 1 mg dialyzed glutamate dehydrogenase was injected intramuscularly without adjuvant to bind the circulating antibodies. This method always prevented anaphylaxis after the booster injection. After 6 h, 4–5 mg dialyzed glutamate dehydrogenase without adjuvant were injected into the auricular vein. 7 days later, the animals were killed by bleeding through the auricular and carotid arteries as described above.

Immunological methods

Immunodiffusion. Gel diffusion as described by OUCHTERLONY[6] and by PIAZZI[7], and immunoelectrophoresis by the method of GRABAR AND WILLIAMS[8] in SCHEIDEGGER'S[9] micro-modification were carried out in 0.2 M Sørensen buffer containing $1 \cdot 10^{-3}$ M EDTA, pH 7.5, in a 1% agar solution. Radial immunodiffusion was performed as described elsewhere[10].

Two-dimensional immunoelectrophoretic analysis according to LAURELL[11] with CLARKE AND FREEMAN'S[12] and LINKE'S[13] modifications was carried out in the first dimension for 30 min in 1% cooled agarose in 0.066 M sodium barbital buffer, pH 8.6, with 200 V and 30 mA. The precipitation gel contained 4 ml each of immune serum and 2% agarose in barbital buffer. In the second dimension, electrophoresis was performed for 14–16 h with 100 V and 45 mA at 4°.

Precipitin curves. Nephelometry followed the method described by SCHULTZE AND SCHWICK[14] in WACHSMUTH'S[15] micro-modification. The precipitates of the Heidelberger curves were measured as described by HEIDELBERGER AND KENDALL[16] with the Folin reagent. Heidelberger curves were obtained with increasing antigen and constant antibody concentrations (α-method) and with increasing antibody and constant antigen concentration (β-method). Nephelometry was carried out at 436 nm in an Eppendorf photometer after incubation for 30 min at 25°. All samples had constant final volumes; they were stored at 4° for 24 h after nephelometry, centrifuged at 10 000 × *g*, and the protein content of the sediment was measured. A blank consisting of rabbit serum before immunization instead of antiserum was carried

along with each sample; this value was subtracted from the protein sediment found after 24 h. An unspecific protein denaturation during the 24-h incubation necessitated this precaution. In the β-method the main value and the blank were determined in a volume of 10 ml per sample, in order to obtain a measurable antigen–antibody precipitate. The final volume of the nephelometric measurement with the α-method was 0.3 ml. The final volumes were achieved by adding 0.2 M Sørensen buffer containing $1 \cdot 10^{-3}$ M EDTA, pH 7.5.

The supernatants of the Heidelberger curves were passed through a column of Sepharose 6B (1.8 cm × 30 cm, Sepharose 6B; 1.8 cm × 2 cm, Sephadex G-25), with proteins of known molecular weight[17] and equilibrated with 0.2 M Sørensen buffer with $1 \cdot 10^{-3}$ M EDTA, pH 7.5. The eluates were collected under photometric control at 280 nm in fractions of 0.3 ml and tested for glutamate dehydrogenase activity.

Passive hemagglutination. The antiserums were titrated by passive hemagglutination by the method of KABAT AND MAYER[18] with modifications by RAJEWSKI *et al.*[19] and PFLEIDERER *et al.*[20], using the micro-titer equipment from Cooke Co., Alexandria.

Biochemical methods

Enzymatic activity of glutamate dehydrogenase was determined as described by SCHMIDT[21] with 2-oxoglutarate and by SUND AND ÅKESON[22] with L-glutamate as substrate. The activity was expressed in I.U. = μmoles substrate per min at 25° and 366 nm. Enzyme-specific staining of the glutamate and lactate dehydrogenases in agar, agarose and starch gels was carried out by the method of FALKENBERG *et al.*[23].

Protein was determined with the biuret reaction by the method of BEISENHERZ *et al.*[24] and with the Folin-reagent according to the procedure of LOWRY *et al.*[25].

Starch gel electrophoresis by the method of SMITHIES[26] was done with the Pherograph Frankfurt-Mini 68 (Hormuth und Vetter, Wiesloch) for 6 h at 1000 V and 45 mA in 11.5% starch gel in 0.03 M borate–NaOH buffer, pH 8.4, and with 0.15 M borate–NaOH buffer, pH 8.4, in the electrode troughs.

Activation and inhibition of glutamate dehydrogenase by its antibodies

(1) 2-oxoglutarate as substrate. 10 μg desalted human glutamate dehydrogenase or bovine glutamate dehydrogenase were dissolved in 0.1 M TRA–HCl buffer, pH 7.6, (the buffer was added until a final volume of 1 ml was reached) and incubated for 15 min at 25° with increasing quantities of antiserum or normal rabbit serum, respectively. 1.75 ml 0.1 M TRA–HCl buffer, pH 7.6, 0.1 ml NADH (10 mg/ml) and 0.1 ml 3.2 M ammonium acetate were added, and the reaction was started with 0.05 ml 0.41 M 2-oxoglutarate. Before adding the antibody in measurements with ADP and GTP, the samples were incubated for 5 min at 25° with ADP or GTP in a concentration of $3 \cdot 10^{-3}$ M so that after complementing the volume to 3 ml the nucleotide concentration was $1 \cdot 10^{-3}$ M at the start of the enzymatic reaction.

(2) L-glutamate as substrate. Activation and inhibition in the reaction with L-glutamate as substrate were performed as described above. The quantity of enzyme used in each test was 50 μg human glutamate dehydrogenase or bovine glutamate dehydrogenase; the buffer was 0.066 M KH_2PO_4, pH 7.8, and the reaction was started by adding 1.6 ml buffer, 0.2 ml NAD^+ (10 mg/ml) and 0.2 ml 1 M L-glutamate. In

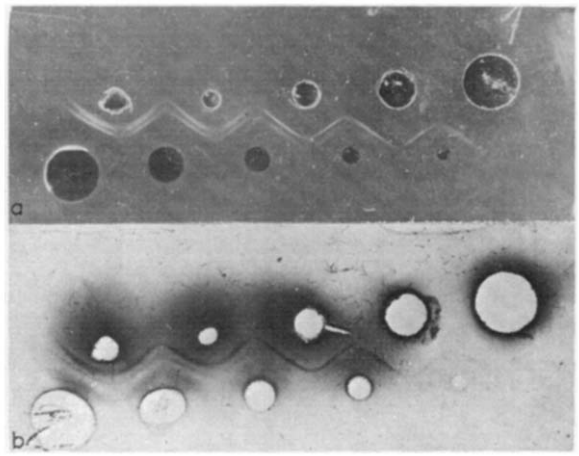

Fig. 1. Piazzi double diffusion. (a) Unstained plate. (b) Enzyme-specifically stained plate. 10 mg/ml human glutamate dehydrogenase as antigen in the upper row, anti-human glutamate dehydrogenase from the rabbit in the lower row.

the allosteric measurements with ADP and GTP the samples were incubated for 5 min at 25° before the antibody was added.

Human glutamate dehydrogenase and bovine glutamate dehydrogenase were stable for months with 40% ammonium sulfate in 0.2 M Sørensen buffer containing $1 \cdot 10^{-3}$ M EDTA, pH 7.4. Before investigations, the enzyme crystals were dissolved and desalted by equilibrium dialysis in Visking bags (Union Carbide Corp., Chicago) for 4 h against a 1000-fold volume of 0.2 M Sørensen buffer with $1 \cdot 10^{-3}$ M EDTA, pH 7.5. Denatured protein was removed by centrifugation at 36 000 $\times$ g. The protein content of the supernatants was determined with the biuret reagent (biuret factor for glutamate dehydrogenase 17.3[1]), and the desired concentration was attained by dilution with buffer as described above.

RESULTS

Immunodiffusion

In OUCHTERLONY[6] double diffusion, in radial immunodiffusion as described by MANCINI in agarose gel containing antibody and in immunoelectrophoresis, several antigen–antibody precipitates between human glutamate dehydrogenase and its antibodies, which contained stabilized enzymatic activity, were found in serial dilutions. In PIAZZI's[7] modification of double diffusion, 4 enzymatically active precipitates were found (Fig. 1). With radial immunodiffusion, up to 5 antigen–antibody precipitates were obtained when a highly concentrated human glutamate dehydrogenase solution (20 mg/ml) and a large antigen reservoir (14 μl) were used.

Two-dimensional immunoelectrophoretic analysis

In two-dimensional immunoelectrophoretic analysis in agarose gel containing antibodies, only one enzymatically active antigen–antibody precipitate was found in low enzyme concentration (Fig. 2c). With increasing enzyme concentration, several enzymatically active antigen–antibody precipitates were found which move toward

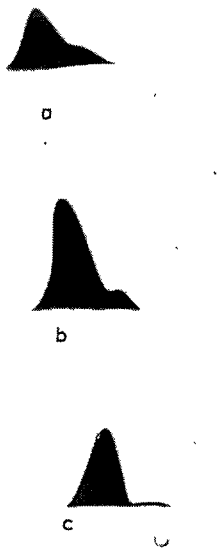

Fig. 2. Laurell electrophoresis. Concentration of the antigen in (a) 10 mg human glutamate dehydrogenase per ml; in (b) 5 mg human glutamate dehydrogenase per ml; in Fig. (c) 1 mg human glutamate dehydrogenase per ml. After electrophoresis in 1% agarose gel in the horizontal direction, 1% agarose containing anti-human glutamate dehydrogenase serum was added and electrophoresis resumed in the vertical direction. The 3 preparations were obtained in the same experiment and enzyme-specifically stained.

the anode (Figs. 2a and 2b). The quantity of antigen–antibody complex did not increase linearly with increasing enzyme concentration. If the planimetrically determined area of the precipitate obtained with 1 mg human glutamate dehydrogenase per ml (2 μg enzyme, Fig. 2c) was taken as 100%, the areas with greater enzyme concentration decreased: an area of only 41.4% was measured with a concentration of 5 mg/ml (10 μg human glutamate dehydrogenase, Fig. 2b) and of only 22.6% with a concentration of 10 mg/ml (20 μg human glutamate dehydrogenase, Fig. 2a). In Fig. 2c, 100% of the total area is united in a single peak; in Fig. 2b, 15% of the total area lies at the same site as in Fig. 2c, and the remaining 85% constitutes a second peak which moves further toward the anode. In the same place as this peak, Fig. 2a shows 11.4% of the precipitated area, 48.7% lies farther toward the anode and 39.9% lies almost at the same place but slightly nearer to the anode.

Precipitin techniques

The nephelometrically measured antigen–antibody complexes and/or the protein precipitates of the Heidelberger curves with the α-method (constant antibody and increasing antigen concentration) were atypical. After a steep rise in the quantity of antigen–antibody precipitates in the range of low antigen concentrations, the Heidelberger curve flattened at higher antigen concentrations (Fig. 3). The antigen–antibody complex is not dissolved by excess of antigen. Up to a concentration of about 1 mg/ml, there was a linear correlation between the antigen–antibody pre-

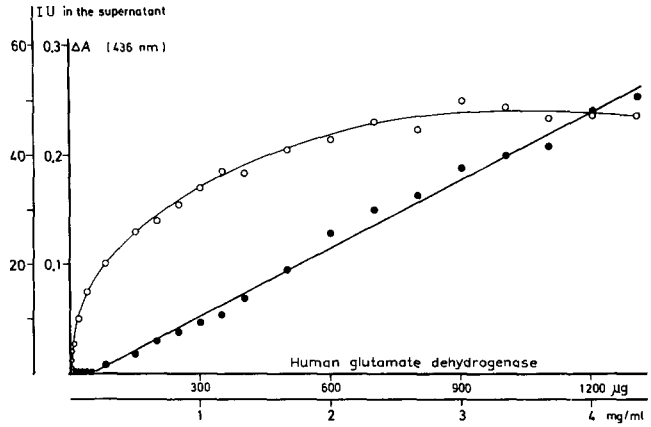

Fig. 3. Nephelometric HEIDELBERGER curve and measurement of the enzymatic activity in the supernatant in the human glutamate dehydrogenase–anti-bovine glutamate dehydrogenase system with constant antibody and increasing antigen concentration (α-method). ○, Protein content measured nephelometrically; ●, enzymatic activity in the supernatant.

cipitate and the antigen added; measurements of the enzymatic activity in the supernatants showed that the equivalence point was situated at about the middle of the ascending slope. Enzymatic activity occurred in the supernatants if human glutamate dehydrogenase was polymerized (above 0.2 mg/ml).

When the principle was inversed and Heidelberger curves were made by nephelometry and/or protein measurement in the precipitate with the β-method (constant antigen and increasing antibody concentration), the curves at first showed the same shape as with the α-method (Fig. 4). At the maximum of the curve, the quantity of precipitated protein exceeded that of the antigen added to the reaction mixture. The quantity of precipitated antigen–antibody complexes was too great to be due to the antibody added. The precipitate increased only slightly when 10 times

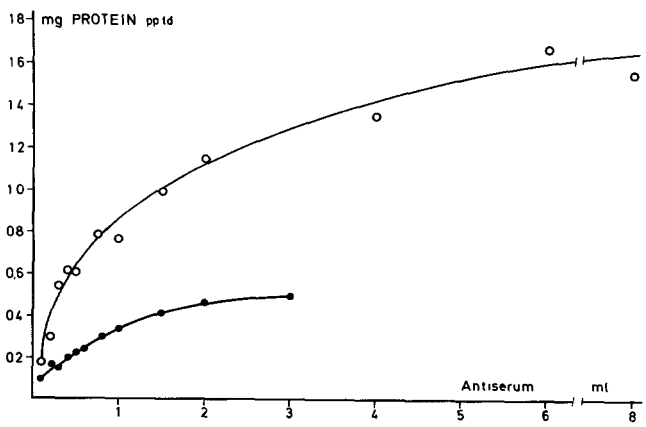

Fig. 4. Quantitative precipitation curves obtained by measuring the protein precipitation with constant antigen and rising antibody concentration (β-method) in the homologous system, human glutamate dehydrogenase–anti-human glutamate dehydrogenase. ○, 0.1 mg/ml human glutamate dehydrogenase; ●, 0.01 mg/ml human glutamate dehydrogenase.

this quantity of antigen was used under otherwise identical conditions (as in Fig. 4). This disproportionate quantity of precipitate was due to protein denaturation in the antiserum. In order to obtain readily measurable antigen–antibody precipitates in low antigen concentrations (1 mg/ml), large volumes (10 ml per sample) with high antiserum content had to be used. Only when a blank (unspecific protein precipitation due to denaturation in antiserum with buffer but without antigen) was subtracted from the main value, the actual antigen–antibody precipitate could be found. With an antigen concentration of 1 mg human glutamate dehydrogenase per ml under these conditions a typical Heidelberger curve with the β-method was found (Fig. 5). After a steep rise in antigen excess and a maximum in the antigen–antibody equivalence range, the curve declined in antibody excess through dissolution of the antigen–antibody complex. With lower antigen concentrations the measured quantity of precipitate was so slight that the magnitude of the blank which was to be subtracted from it rendered the spread of the values too great. Higher antigen concentrations (and thus polymeric human glutamate dehydrogenase) again give Heidelberger curves of the type shown in Figs. 3 and 4.

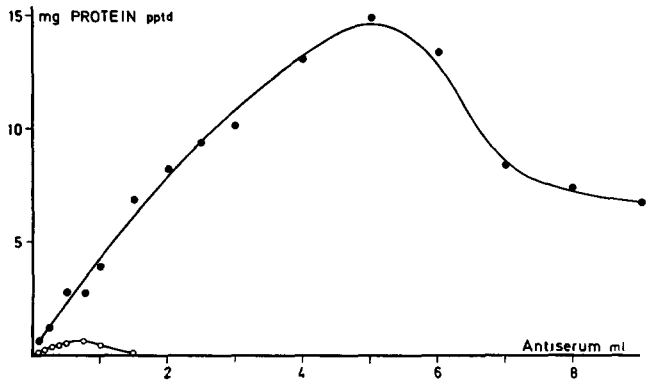

Fig. 5. Quantitative precipitation curve with constant antigen and increasing antibody concentration (β-method) in the human glutamate dehydrogenase–anti-human glutamate dehydrogenase system after subtracting the precipitation blank for every sample after adding non-immune rabbit serum in place of the rabbit serum with antibodies. ○, 0.1 mg/ml human glutamate dehydrogenase; ●, 1 mg/ml human glutamate dehydrogenase.

When the procedure was repeated under the conditions indicated in Fig. 5 and the precipitate measured after 24 h, further antigen–antibody precipitate was obtained by centrifuging the samples and substracting the above-mentioned blanks after 6 and 12 days. The form of the curves of the antigen–antibody complex precipitated between the 1st and the 6th day and between the 6th and the 12th day showed an increase in the antigen–antibody complex precipitated in the samples with high antigen content. After removal of the mainly monomeric antigen by its antibody during the first 24 h, the concentration declined in the incubation mixture; further dissociation of the human glutamate dehydrogenase to the monomeric form produced new precipitates in the range of antibody excess.

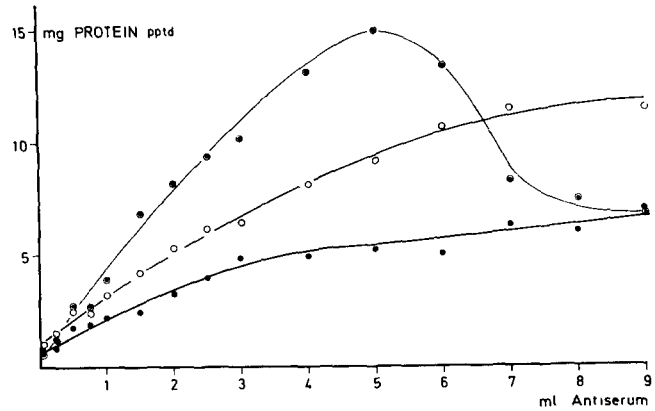

Fig. 6. Quantitative precipitation curve after 24 h, after 6 days and 12 days with constant antigen (1 mg human glutamate dehydrogenase per ml) and rising antibody concentration (β-method) in the human glutamate dehydrogenase–anti-human glutamate dehydrogenase system. ○, Protein precipitated after 24 h, ○, protein precipitated between 24 h and the 6th day, ●, protein precipitated between the 6th and 12th day.

Elimination of soluble antigen–antibody complexes

High-voltage starch gel electrophoresis of the supernatants of the Heidelberger curves with excess of antigen, antigen–antibody equivalence and excess of antibody showed several "enzyme-specifically" stained bands with increasing antibody concentration (Fig. 7a). Equally strong staining of these additional bands could, however, also be obtained by incubating the samples without substrate (Fig. 7b). The use of L-lactate instead of L-glutamate produced the artifacts described by FALKENBERG *et al.*[23] in so-called enzyme-specific staining; the free lactate dehydrogenase isoenzymes

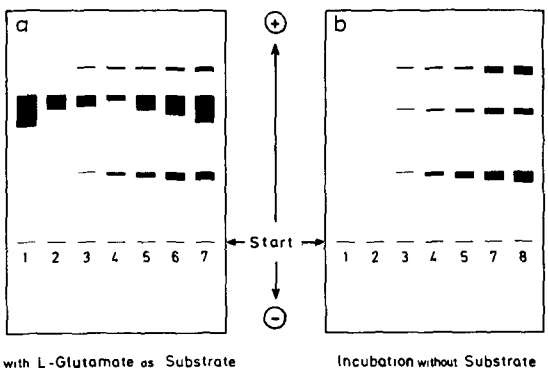

Fig. 7. Starch gel electrophoresis with "enzyme-specific" staining with L-glutamate as substrate and blank staining without substrate. Sample 1, dialyzed, pure human glutamate dehydrogenase. Samples 2–7 are supernatants of the Heidelberger curves with antigen excess (Samples 2 and 3), antigen–antibody equivalence (Sample 4) and antibody excess (Samples 5–7). In the incubation without substrate, Sample 8 is non-immune rabbit serum. The Heidelberger curve was made with constant antigen (1 mg human glutamate dehydrogenase per ml) and increasing antibody concentration (β-method). The staining of the bands without substrate was intensified by the addition of L-lactate. Greater quantities of free antigen occur in the range of antibody excess. Soluble antigen–antibody complexes without stabilized enzymatic activity are not formed.

in the rabbit serum gave rise to these bands (Fig. 7b, No. 8). Free glutamate dehydrogenase could not be detected in rabbit serum. The human glutamate dehydrogenase band in the middle of the gel block showed a continuous decrease in the range of antigen excess up to antigen–antibody equivalence; the antigen–antibody precipitates were then dissolved in the range of antibody excess.

Gel chromatography of the supernatants of the Heidelberger curves on Sepharose 6B columns calibrated for molecular weight determinations[17] also failed to show the presence of soluble antigen–antibody complexes (Fig. 8). Fully active monomeric human glutamate dehydrogenase appeared at a molecular weight of about 300 000 and polymeric human glutamate dehydrogenase at a molecular weight of about $2 \cdot 10^6$ in the eluate; soluble antigen–antibody complexes consisting of dimers and antibodies, trimers and antibodies, *etc.* emerged from the column between these molecular weights; a soluble antigen–antibody complex formed by the antibodies and the highly polymeric aggregate with a molecular weight of more than $2 \cdot 10^6$ left the Sepharose column before the polymeric human glutamate dehydrogenase. In the presence of an excess of antigen or in the equivalence range (Fig. 8a), only the polymeric enzyme was eluted. With excess of antibody (Fig. 8b), only the polymeric enzyme was eluted. With excess of antibody (Fig. 8b), only one human glutamate dehydrogenase peak was found in the same position; this peak was higher than that found in the equivalence range. Chromatography of rabbit serum (Fig. 8c) showed no enzymatic activity in the eluate, as was to be expected. The elution diagram showed that the difference between the protein curves for antigen excess and antibody

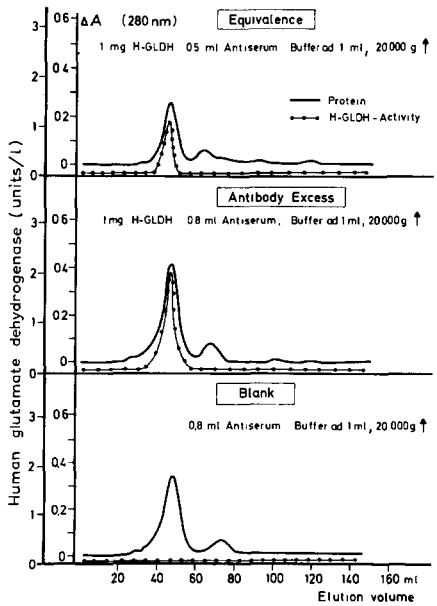

Fig. 8. Gel chromatography on Sepharose 6B of the supernatants of the Heidelberger curve (system human glutamate dehydrogenase–anti-human glutamate dehydrogenase with an antigen concentration of 1 mg/ml (β-method)), in antigen–antibody equivalence (a) and in antibody excess (b). (c) Gel filtration of rabbit blank serum. Only one enzymatically active macromolecular human glutamate dehydrogenase peak is formed; soluble antigen–antibody complexes are not eluted.

excess were due to the higher content of rabbit serum added. The existence of soluble antigen–antibody complexes with the different macromolecular polymeric aggregation states of human glutamate dehydrogenase could not be demonstrated.

Activation of glutamate dehydrogenase by its antibodies

When rabbit serum which did not contain antibodies was incubated with dissolved human glutamate dehydrogenase and bovine glutamate dehydrogenase, these enzymes were unspecifically activated by 15–70% in the reaction with 2-oxoglutarate and L-glutamate as substrates when the enzymatic activity without serum was taken as 100%.

In the reaction with 2-oxoglutarate as substrate, anti-human glutamate dehydrogenase activated human glutamate dehydrogenase to 227% and bovine glutamate dehydrogenase to 340% of the original value. Anti-bovine glutamate dehydrogenase serum activated its homologous antigen to 384% and human glutamate dehydrogenase to 215% of the original value (Fig. 9a). Both serums activated

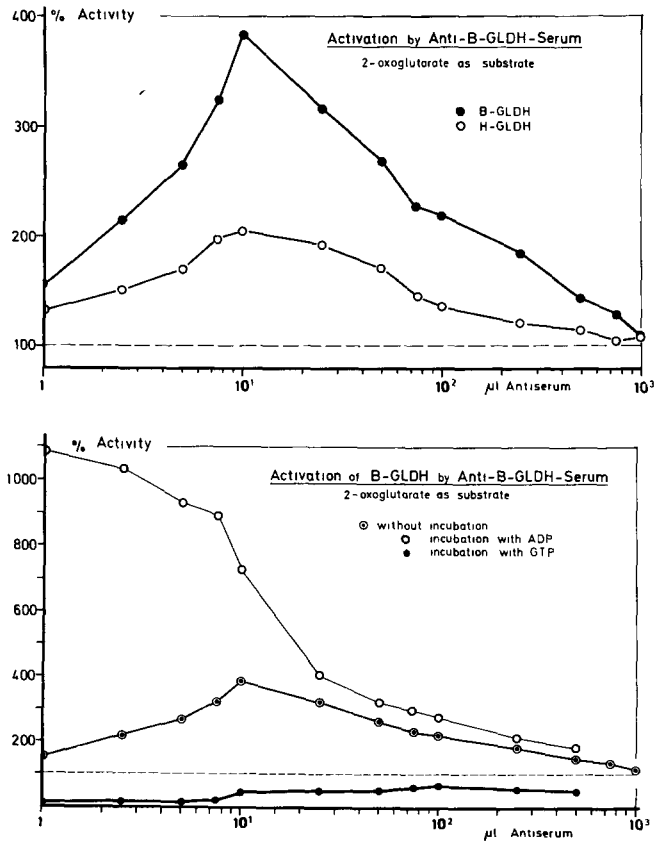

Fig. 9. (a) Activation of human glutamate dehydrogenase and of bovine glutamate dehydrogenase by anti-bovine glutamate dehydrogenase serum with 2-oxoglutarate as substrate. (b) Competitive inhibition between ADP, GTP and bovine glutamate dehydrogenase antibodies in the bovine glutamate dehydrogenase–anti-bovine glutamate dehydrogenase system. Concentration of nucleotides $1 \cdot 10^{-3}$ M.

bovine glutamate dehydrogenase more than human glutamate dehydrogenase. The activation of human glutamate dehydrogenase and bovine glutamate dehydrogenase was slightly greater when the homologous antiserums were used. The maximal activation of both glutamate dehydrogenase proteins was attained with anti-bovine glutamate dehydrogenase serum at a 100-fold lower concentration than with anti-human glutamate dehydrogenase serum. Antibody titration as described by RAJEWSKI *et al.*[19] was not possible because of the dissociation effect of human glutamate dehydrogenase and bovine glutamate dehydrogenase in progressive dilution series and because of the different binding capacities of the various dissociation products of the glutamate dehydrogenases for their antibodies. Under identical conditions the antibody titers of the two antiserums (anti-human glutamate dehydrogenase serum and anti-bovine glutamate dehydrogenase serum) were equal in passive hemagglutination.

In the enzymatic reaction with 2-oxoglutarate as substrate, the allosteric activation of the bovine glutamate dehydrogenase–anti-bovine glutamate dehydrogenase system (Fig. 9b) by ADP was partially reversed by the later addition of

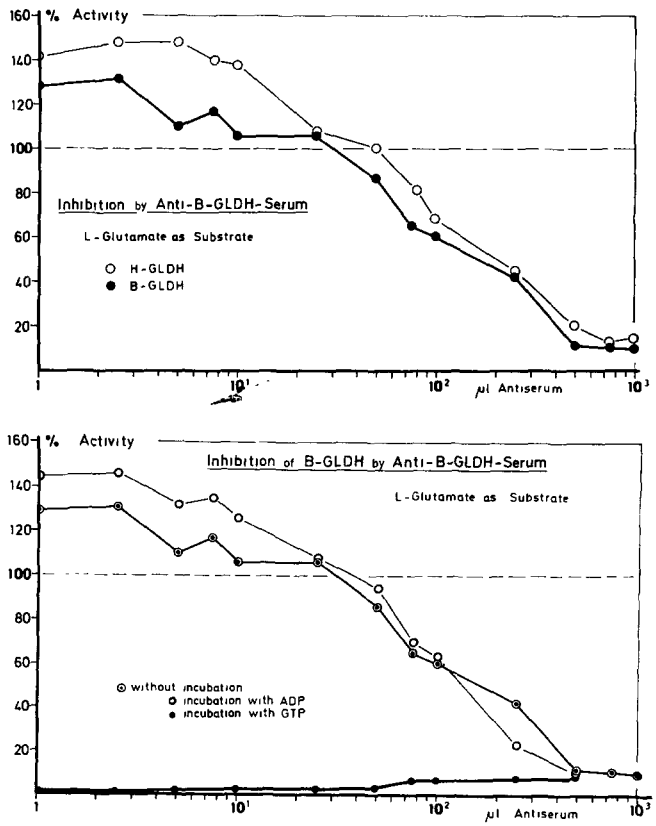

Fig. 10. (a) Inhibition of human glutamate dehydrogenase and of bovine glutamate dehydrogenase by anti-bovine glutamate dehydrogenase serum with L-glutamate as substrate. (b) Competitive inhibition between ADP, GTP and bovine glutamate dehydrogenase–antibodies in the bovine glutamate dehydrogenase–anti-bovine glutamate dehydrogenase system. Concentration of nucleotides $1 \cdot 10^{-3}$ M.

antibody in the concentration range in which the enzyme was activated by its antibody. The almost complete allosteric inhibition by GTP would under these conditions, reach 70–80% of the original activity in the range of antibody activation. In the human glutamate dehydrogenase–anti-human glutamate dehydrogenase system there was also inhibition of the allosteric activation by ADP. The allosteric inhibition by GTP in the range of the maximal activation by the antibody is also partially reversed. As in the reaction without allosteric effectors, anti-human glutamate dehydrogenase serum was less effective than anti-bovine glutamate dehydrogenase serum when both sera had equal antibody titers.

Inhibition of glutamate dehydrogenase by its antibodies

In the reaction with L-glutamate as substrate, the antibodies to the human enzyme led only to a slight inhibition of the activity of human glutamate dehydrogenase and bovine glutamate dehydrogenase, even when the antibody was present in extreme excess. Anti-bovine glutamate dehydrogenase serum, on the other hand, inhibited the heterologous human and the homologoes bovine protein with excess of antibody to below 10% of the original value (Fig. 10a). The inhibition of human glutamate dehydrogenase by its heterologous antibody was almost as pronounced as the inhibition of bovine glutamate dehydrogenase in the homologous system, although only about 50% of the antibody to the animal enzyme could take effect on the surface of the human protein[3].

In the reaction with L-glutamate as substrate, antibodies to human glutamate dehydrogenase could not cause a change in the allosteric activation of ADP or in the allosteric inhibition by GTP either in the human or in the animal protein. The antibody to bovine glutamate dehydrogenase reversed the allosteric activating effect of ADP completely and the inhibiting effect of GTP almost completely with human glutamate dehydrogenase as well as with bovine glutamate dehydrogenase. These results are analogous to the findings with anti-bovine glutamate dehydrogenase serum in the reversed reaction with 2-oxoglutarate as substrate.

DISCUSSION

Crystalline glutamate dehydrogenases from human and bovine liver showed atypical immunological reactions with their antibodies in the homologous and heterologous systems in gel diffusion, in one- and two-dimensional immunoelectrophoresis, in quantitative precipitation curves and also when the enzymes were activated and inhibited by the addition of the antibodies. Human and bovine glutamate dehydrogenase probably had no antigenic determinants at the active center or in its immediate vicinity: (1) Antigen–antibody precipitates contained stabilized enzymatic activity which was not directly influenced by the reaction between the antigen and antibodies. (2) The specific activity of glutamate dehydrogenase with L-glutamate as substrate probably did not change during polymerization (FRIEDEN AND COLMAN[27]); thus it seems that during polymerization, the catalytic centers were situated at readily accessible places in the protein molecule on the outside of the polymeric enzyme complex. The antibody-binding sites of human glutamate dehydrogenase and bovine glutamate dehydrogenase were, however, not readily accessible in the polymeric enzyme complex. (3) Also, the activation in the

reaction with 2-oxoglutarate as substrate almost completely eliminated the possibility that the macromolecular antibody whose size contrasted with the small coenzyme and substrate molecules, was bound in the immediate vicinity of the active center. (4) The peptide Gly^{94} to Lys^{105}, obtained from bovine glutamate dehydrogenase (SMITH *et al.*[28]) by trypsinizing the enzyme after binding radioactively labeled pyridoxal phosphate to the active center (Lys^{97}), contained no phenylalanine, tryptophan or tyrosine; the absence of aromatic amino acids in this part of the molecule could also be an indication of the absence of antigenic determinants.

In comparitive studies, we found no differences in the dissociation and association behavior between human glutamate dehydrogenase and bovine glutamate dehydrogenase; human glutamate dehydrogenase underwent a graduated dissociation which was dependent on the concentration. This could be shown qualitatively in serial dilutions (double diffusion according to the procedure of OUCHTERLONY[6], immunodiffusion as described by PIAZZI[7] and immunoelectrophoresis), semiquantitatively (MANCINI technique and quantitative precipitation) and quantitatively (2-dimensional immunoelectrophoretic analysis) by immunological methods. Antibodies to human glutamate dehydrogenase precipitated only monomeric human glutamate dehydrogenase quantitatively. Increasing concentration and the correspondingly increasing polymerization did not lead to a linear rise in the quantity of antigen-antibody complex precipitated. The rabbits were immunized by injecting polymeric human glutamate dehydrogenase in a solution or emulsion with a concentration of 5 mg/ml. After resorption of the intraplantarly, intramuscularly, subcutaneously or intravenously administered solution or emulsion, the human glutamate dehydrogenase was probably dissociated through dilution to the monomeric form before it reached the immune cells of the rabbits, so that the antibodies were directed against the monomeric enzyme protein. The ultrasonic treatment used to emulsify the salt-free antigen with complete Freund's adjuvant may have furthered dissociation: decreased molecular weight of salt-free, ultrasonically treated human glutamate dehydrogenase and bovine glutamate dehydrogenase, which were not denatured, could be shown by chromatography of the enzymes in 0.2 M Sørensen buffer with $1 \cdot 10^{-3}$ M EDTA, pH 7.5, on Sepharose 6B columns calibrated for molecular weight[3]. We were not able to observe precipitation of highly polymerized human glutamate dehydrogenase by its antibodies. Molecular sieve electrophoresis in starch gel and gel chromatography on columns of Sepharose 6B calibrated for molecular weight were used to show that macromolecular, soluble antigen–antibody complexes were not present either with antigen excess, in the range of equivalence or with antibody excess. The unusual immunological behavior shown by our antigen–antibody complexes in immunodiffusion, immunoelectrophoresis and in quantitative precipitation curves could be explained when one assumes that rabbit antibodies were directed only against those protein surfaces of the monomer which became "buried" inside the molecule when it was polymerized. In contrast to monomeric human glutamate dehydrogenase, the dimeric form could only be partially precipitated, as only some of the antigenic determinants were still available to the antibody. The number of antibody-binding sites decreased progressively in the trimeric, tetrameric and more highly polymeric forms. The several enzymatically active antigen–antibody precipitates, up to five in number, which occurred in one enzyme–anti-enzyme system between human glutamate dehydrogenase and its antibodies,

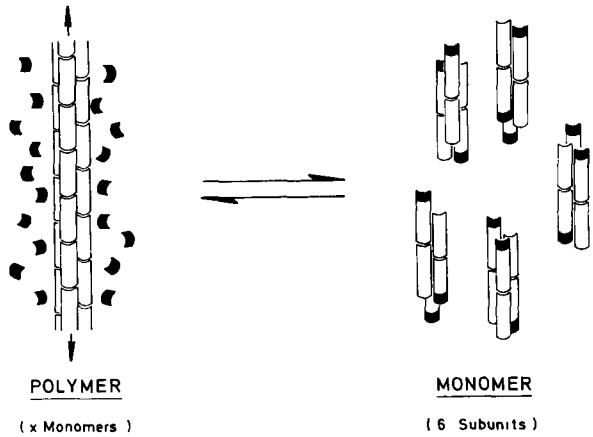

Fig. 11. Application of our results to EISENBERG AND REISLER's[29] newest glutamate dehydrogenase model as modified according to the results of SUND (unpublished). The smallest, still enzymatically active unit (molecular weight, 312 000) consists of 6 identical, inactive subunits (molecular weight, 52 000), which are probably cylindroids interlocked with each other. An unlimited number of monomers associates to a polymer of chain form. The antibodies are bound at sites which are concealed in the polymeric state.

could by understood as complexes formed of the antibodies and the monomeric, dimeric, *etc.* glutamate dehydrogenase-proteins, respectively.

The concealment of free antibody-binding sites of human glutamate dehydrogenase when the enzyme was polymerized explained the atypical Heidelberger curves made with the α-method, *i.e.* with constant antibody and increasing antigen concentration. Only in the range of low antigen concentrations in which human glutamate dehydrogenase was present mainly in the monomeric form, could typical Heidelberger curves be observed. Heidelberger curves made with the β-method, *i.e.* with constant antigen and increasing antibody concentration, were of typical form when the antigen concentration was low and the enzyme predominantly monomeric. The precipitation of dissolved polymeric human glutamate dehydrogenase in the presence of antibody excess after changing the dissociation equilibrium by removing precipitated antigen–antibody complexes after several days also indicated that the antigenic determinants were situated on the protein surfaces which were exposed only in the monomeric state of the enzyme and that there were no soluble, highly polymerized antigen–antibody complexes. When our results were applied to EISENBERG AND REISLER's[29] newest bovine glutamate dehydrogenase model, as shown diagrammatically in Fig. 11, incorporating SUND's results, it appeared likely that the antibody-binding sites were located at the points of connection between the monomers in polymerization.

Thus there were no free antibody–binding sites at or near the active center, the activation of human glutamate dehydrogenase and bovine glutamate dehydrogenase in the reaction with 2-oxoglutarate as substrate and their inhibition in the reaction with L-glutamate as substrate could not be explained with KOSHLAND's[30] "induced fit"-mechanism, *i.e.* a conformation change caused by the action of antibodies in the region of the active center. Several results supported the hypothesis that there were antibody–binding sites in the area of the allosteric sites of ADP

and of GTP on the enzyme surface. The reversibility of the allosteric activation by ADP or of the allosteric inhibition by GTP by later addition of antibody indicated competition between allosteric effectors and antibodies. A dissociation of polymeric human glutamate dehydrogenase or bovine glutamate dehydrogenase complexes by the antibodies which carried its antigenic determinants inside the polymeric complex, could not explain this phenomenon. An interpretation of the antibody effect in both directions of reaction as an allosteric effect of the antigenic determinants in the region of these sites seemed feasible, firstly because of the known closeness between the allosteric sites for ADP activation and for GTP inhibition[27,31], and secondly, because of the size of the antibody compared to the allosteric effectors. In the primary structure of bovine glutamate dehydrogenase aromatic amino acids were relatively numerous in the region of Tyr^{412}; this tyrosine was labeled with nitro compounds by SMITH *et al.*[28] according to the method used by DI PRISCO to localize the allosteric site of GTP inhibition; these aromatic amino acids could further the formation of immunogenic determinants in this region. However, these considerations were only speculative as long as the tertiary structure of human glutamate dehydrogenase and bovine glutamate dehydrogenase is not known. This activation and inhibition of the enzymatic activity and the simultaneous competition with allosteric effectors, observed in our results for the first time in one enzyme–anti-enzyme system, could be explained by hitherto known mechanisms of activation and inhibition by antibodies[3,32–36]. The partly different findings reported by FAHIEN *et al.*[5] in the frog glutamate dehydrogenase–anti-frog glutamate dehydrogenase system and by CORMAN AND KAPLAN[37] in the chicken glutamate dehydrogenase or bovine glutamate dehydrogenase–anti-bovine glutamate dehydrogenase system may be due to the higher antibody content of our antiserums and to our consideration of the unspecific activation caused by rabbit serum, which was not yet known to these authors. Further investigations must be made in order to elucidate the mechanism of this phenomenon and to discover whether a single antibody population or different antibodies were responsible for the effects described above.

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