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The Interaction between α_2-Macroglobulin and Cationic Aspartate Aminotransferase

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On starch-gel or polyacrylamide-gel electrophoresis of human serum, a supernumerary zone of aspartate aminotransferase activity may be demonstrated, migrating with the slow α_2 protein zone. This appearance is due only to cationic aspartate aminotransferase, bound by α_2-macroglobulin. The binding is strongly potentiated by dilute borate buffers.

Aspartate aminotransferase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1) comprises anionic and cationic isoenzymes, which are found respectively in the soluble fraction of the cell and in the mitochondria (Fleisher, Potter & Wakim, 1960; Rosenthal, Thind & Conger, 1960). Electrophoretic subfractions of each have been described (Martinez-Carrion *et al.* 1967; Martinez-Carrion & Tiemier, 1967), but this type of heterogeneity is unrelated to the phenomenon discussed below.

If serum, or a mixture of serum with an extract of heart, liver or kidney, is subjected to electrophoresis in starch gel or polyacrylamide gel, a distinct, slow-moving, anionic zone of aspartate aminotransferase activity may be demonstrable, coincident with the 'slow α_2' protein zone (Boyde & Latner, 1962; Boyde, 1968*b*). This zone never appears in tissue extracts in the absence of serum, and is thus presumably the result of interaction of a serum factor with an enzyme or enzyme precursor. The experiments described below were intended to identify the serum and tissue factors, and to establish the nature of their interaction.

METHODS

Assay. Aspartate aminotransferase activity was assayed as described by Boyde (1968*a*). To study the effect of α_2-macroglobulin on the activity of cationic isoenzyme, the experimental conditions were altered by substituting appropriate buffers (with or without added α_2-macroglobulin) for the tris–acetate buffer used in the standard assay, and by using lower concentrations of the substrates.

Preparation of isoenzyme concentrates. The isoenzymes were separated and partially purified as described by Boyde (1968*a*).

Polyacrylamide-gel electrophoresis. Electrophoresis and detection of zones of enzyme activity were by methods described by Boyde (1968*b*). Gels were subsequently stained for protein with Naphthalene Black, differentiated by soaking alternately in water and methanol–acetic acid–water (5:1:5, by vol.), and photographed with a Kodak no. 61 filter.

The specificity of the detection procedure for aspartate aminotransferase was checked by running gels in triplicate, with two of these used for 'blank' experiments in which one of the substrates was omitted (L-aspartate in one, 2-oxoglutarate in the other). No zone of activity appeared in any such blank experiment.

Gel-filtration chromatography. All operations were carried out at 4°. The column used was a Perspex chromatographic column (LKB) of 3·6cm. internal diam. (10cm.^{2} cross-sectional area), filled with fully swollen Sephadex G-150 (Pharmacia, Uppsala, Sweden) to a height of 76cm. Retrograde flow of eluent was provided by a peristaltic pump (LKB) at 13ml./hr., and the eluate collected as 5ml. fractions. The column was equilibrated with the buffer to be used by passing this through for 24hr. before applying the sample, and the sample itself was prepared by dialysing overnight against the buffer to be used. The extinction of the eluate was recorded continuously at 257mμ (Fig. 1).

The same column was used repeatedly, and the elution pattern of serum proteins was found to be highly reproducible, so that valid comparisons may be made between parallel experiments.

DEAE-cellulose chromatography. Columns were prepared with 5g. of DE11 (Whatman) packed into a volume of 30ml. and equilibrated with 8mM-sodium phosphate buffer, pH7·0. Elution was with the series of eluents described by Hess & Walter (1960) or with a linear concentration gradient between 10mM-sodium phosphate buffer, pH6·0, and 0·2M-sodium phosphate buffer, pH6·0, over a total volume of 400ml. The flow rate was 10ml./hr., and 5ml. fractions were collected. All operations were conducted at 4°.

RESULTS

Cationic isoenzyme required to produce 'zone II'. When subjected to polyacrylamide-gel electrophoresis in admixture with serum, cationic isoenzyme gave rise to a distinct zone of activity, coincident with the slow α_2 protein zone, and referred to hereafter as 'zone II'. Even at a concentration of 3·7units/ml., pure anionic isoenzyme produced no appearance suggestive of zone II.

As prepared by one stage of DEAE-cellulose

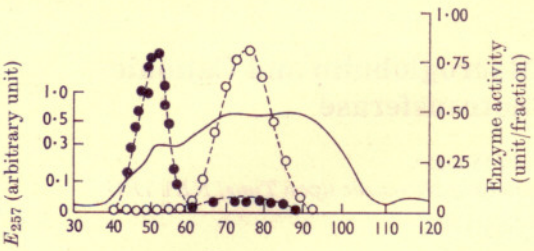

Fig. 1. Sephadex G-150 chromatography of normal human serum. ——, Serum alone, 5 ml. in 0·1 M-sodium phosphate buffer, pH 7·4 (E_{257}). An electrophoretic test for capacity to bind cationic aspartate aminotransferase was carried out as described in the text, and gave positive results from fraction 40 to 63 inclusive. ○--○, 4 ml. of serum plus 14 units of cationic isoenzyme, 0·1 M-sodium phosphate buffer, pH 7·4 (aspartate aminotransferase activity). ●--●, 4 ml. of serum plus 8 units of cationic isoenzyme, 25 mM-borate buffer, pH 8·4 (aspartate aminotransferase activity).

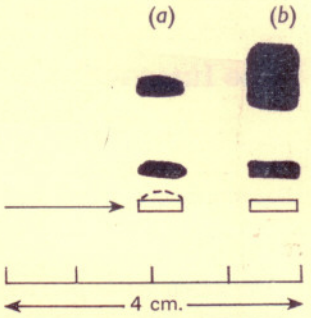

Fig. 2. Polyacrylamide-gel electrophoresis of a mixture of cationic isoenzyme with purified α_2-macroglobulin (tracings from photographs). (*a*) Aspartate aminotransferase activity by the method of Boyde (1968*b*). (*b*) Protein stain (see the text). Origin slots are indicated by the arrow. The gel was arranged anode end uppermost. In the absence of α_2-macroglobulin this isoenzyme migrates towards the cathode.

chromatography (fraction IV; Boyde, 1968*a*), anionic isoenzyme was contaminated with 0·6% of cationic isoenzyme. This was readily detected by polyacrylamide-gel electrophoresis in admixture with serum and was estimated after carrying through a further stage of DEAE-cellulose chromatography (Hess & Walter, 1960), which also yielded the pure anionic isoenzyme referred to above.

Migration of zone II relative to anionic isoenzyme. The ratio between the mobilities of zone II and the major anionic zone of activity was determined at various concentrations of acrylamide: 4% acrylamide, 0·48; 4·5% acrylamide, 0·35; 5% acrylamide, 0·22. On the basis of these results, 4·5% acrylamide was selected as the most suitable for demonstrating zone II. At less than 4% acrylamide zone II was too close to the major anionic zone of activity; at more than 5% acrylamide it was too close to the origin slot.

The absolute mobility of the major anionic zone of activity was relatively little affected by these changes in gel strength, being diminished by 22% in passing from 4% to 5% acrylamide, compared with 63% for zone II.

Identification of serum factor. In a series of early qualitative experiments it was established that zone II and the slow α_2 band were coincident on starch gel, and that only material eluted from the slow α_2 band gave rise to zone II in admixture with tissue extracts (Boyde, 1967). This was followed up by partial purification of the factor responsible.

A 5 ml. sample of normal human serum was subjected to Sephadex G-150 chromatography in 0·1 M-phosphate buffer, and the effluent fractions were tested by mixing 5 vol. with 1 vol. of a preparation of cationic aspartate aminotransferase containing 2 units/ml. and using these mixtures as samples for polyacrylamide-gel electrophoresis. Only the leading (macroglobulin) peak showed the capacity to bind cationic isoenzyme and thus produce a zone II (Fig. 1). Fractions 40–55 were combined, concentrated by ultrafiltration and subjected to DEAE-cellulose chromatography with gradient elution. The fractions eluted between 130 and 200 ml. were combined and concentrated by ultrafiltration. Polyacrylamide-gel electrophoresis showed that more than 90% of the protein in this preparation was in a single intense band with mobility identical with that of the slow α_2 band in normal serum. On mixing with cationic isoenzyme, a very intense zone II was produced, identical in mobility with this major protein zone.

A further purification by preparative polyacrylamide-gel electrophoresis decreased contaminating proteins to trace amounts. Once again this material proved capable of producing a very intense zone II, identical in mobility with the major protein zone.

Binding of purified α_2-macroglobulin. Purified α_2-macroglobulin (a gift from Professor J. W. Mehl, University of Southern California) was mixed with cationic isoenzyme and subjected to polyacrylamide-gel electrophoresis. This preparation of α_2-macroglobulin exhibited two protein bands, probably because of polymerization (Demaille, Dautrevaux, Havez & Biserte, 1965). Cationic isoenzyme was bound to both (Fig. 2).

Binding demonstrated by gel filtration. A 5 ml. sample of normal human serum (mixed with cationic isoenzyme) was subjected to Sephadex G-150 chromatography in 25 mM-borate buffer,

pH 8·4. More than 95% of the enzymic activity was eluted in the leading (macroglobulin) peak (Fig. 1). In 0·1 M-phosphate buffer this did not occur; indeed the elution volume of the enzymic activity was very close to that found in the absence of serum, with the same column (cationic isoenzyme, 382 ml.; anionic isoenzyme, 374 ml.; Boyde, 1967).

Effect of α_2-macroglobulin on activity. Cationic isoenzyme activity was measured in the presence and absence of α_2-macroglobulin (0·167 mg./ml.). In 25 mM-borate buffer, pH 8·4, with 3·3 mM-L-aspartate and 0·33 mM-2-oxoglutarate, α_2-macroglobulin produced a 35% slowing of the reaction rate. There was no such effect with these substrate concentrations in other buffers (tris–acetate, pH 7·4, 33 mM; sodium phosphate, pH 7·4, 33 mM; tris–hydrochloric acid, pH 8·6, 33 mM) or with no added buffer, and no such effect in borate buffer with 100 mM-L-aspartate and 6·7 mM-2-oxoglutarate, though the actual reaction rate differed according to the buffer and the substrate concentrations used.

DISCUSSION

The results set out above strongly indicate that cationic aspartate aminotransferase is bound to α_2-macroglobulin in dilute borate buffer, and that this interaction is solely responsible for the phenomenon referred to as 'zone II'. The formation of zone II cannot be mimicked by excess of anionic isoenzyme, and it seems very unlikely that it can appear because α_2-macroglobulin activates some component of the cationic isoenzyme preparation. Zone II can be demonstrated in normal serum or plasma if incubation is continued long enough, and it has been shown that this, too, is due to the presence of cationic isoenzyme (Boyde, 1968*b*).

Significant binding occurs in the absence of borate and in physiological conditions (Boyde & Pryme, 1968), but borate increases the interaction and is necessary for its demonstration under the peculiar conditions of electrophoresis.

The marked change in mobility of zone II between 4% and 5% acrylamide is additional evidence that it is associated with a macroglobulin. Indeed these results can be used to calculate an approximate molecular weight of 1 000 000 for the zone II complex (Tombs, 1965; M/D taken as 0·5 at 4% acrylamide).

The importance of these findings is, first, that the zone II phenomenon can be relied upon in identifying cationic isoenzyme, and can be employed as a semi-quantitative assay (Boyde, 1968*b*); secondly, as evidence for the binding by α_2-macroglobulin of a protein other than a protease. It was formerly thought that insulin and growth hormone were bound by α_2-macroglobulin, but this is probably not the case (Berson & Yalow, 1966; Keehan, Smith, Howard, Mehl & Beigelman, 1967). All the proteins now known to be susceptible to binding by α_2-macroglobulin are cationic, and this leads to the suggestion that binding is the result of electrostatic interaction rather than any specific effects. α_2-Macroglobulin is a glycoprotein (Schulze, Schmidtberger & Haupt, 1958; Demaille, Dautrevaux, Havez & Biserte, 1966), so borate presumably acts by forming complexes with sugar residues, thus increasing the net negative charge on the molecule.

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