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ISOELECTRIC FOCUSING OF CARBOXYPEPTIDASE N

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Summary

Carboxypeptidase N was partially purified on a TEAE-cellulose column and subjected to isoelectric focusing in sucrose gradient columns containing ampholine gradients of pH range 3—10 and 4—8. Activity separated into two major peaks with p*I* values of pH 3.8 and 4.3. Both peaks were totally converted to an active desialated enzyme with isoelectric point of pH 5.2 to 5.4. These results indicate that carboxypeptidase N is a sialoprotein with at least two forms, differing in sialic acid content, in serum. Catalytic activity is not dependent upon sialic acid but the latter may possibly influence stability since loss of activity occurred in the desialated enzyme with repeat focusing.

Introduction

Carboxypeptidase N is the major enzyme responsible for cleaving basic carboxy terminal amino acids from peptides in serum [1]. It has been shown to inactivate bradykinin and kallidin [2,3], C_3a and C_5a [4] and therefore exerts an important modulating influence on both the kinin and complement systems. Serum activity is reduced in anaphylactic [1] and endotoxic [5] shock, the Dengue shock syndrome [6] and in cirrhosis of the liver [7], and is increased during pregnancy [5,7]. It has been postulated [8,9] that deficient activity could account for the accumulation of positively charged peptides with ciliostatic activity in cystic fibrosis. We have recently found evidence of reduced carboxypeptidase N in the serum of these patients [10].

Since it is possible that genetic factors and/or differential organ synthesis might have an important bearing on serum carboxypeptidase N activity the present study was initiated to determine whether isoenzymes were separable by the isoelectric focusing technique. In this paper we provide evidence that the enzyme is a sialoprotein consisting of at least two species which appear to differ principally with respect to sialic acid content.

Material and Methods

TEAE cellulose chromatography. TEAE 0.87 mequiv./g (Sigma) was equilibrated with 0.02 M potassium phosphate buffer pH 7.0, and loaded into a column 2.5 × 50 cm. The column was exhaustively eluted with the same buffer.

Isoelectric focusing. A Uniphor 7900 column electrophoresis system, (LKB-Produkter AB, Bromma, Sweden), adapted with 35-ml and 110-ml columns was loaded with 5—50% sucrose density gradients containing 1% (w/v) ampholine carrier ampholites. Ampholine gradients (LKB-Produkter AB) were used as commercially available, or by appropriate mixing of commercially available products. Columns were focused for periods of 24 h to 72 h at 200 V initially and 600 V to a maximum of 0.6 W for the 35 ml column and a terminal voltage of 1000, maximum of 1.0 W, for the 110 ml column. At the termination of the focusing run the columns were emptied in fractions of 1.5 to 3.0 ml. The pH of the fractions was immediately measured at room temperature and the fractions rapidly immersed in ice water. In order to preserve enzyme activity it was found to be mandatory to dialyze each fraction within 1 h of sample collection. Each sample was dialyzed versus running tap water for 4 h double distilled water overnight and two changes of 0.02 M potassium phosphate buffer pH 7.0 containing 0.1 mM $CoCl_2$ for 24 h at 4°C. Prior to assay for carboxypeptidase N activity each fraction was reconstituted to its original volume with the last solution.

Neuraminidase treatment. Pooled and dialyzed carboxypeptidase N solutions (approximately 5.0 U) were obtained by TEAE-cellulose chromatography or isoelectric focusing, incubated in a total volume of 5.0 ml containing neuraminidase from *Clostridium perfringens* (0.065 U) (Sigma or protease free, gift of H. Schachter) in 0.02 M potassium phosphate buffer pH 7.0 at 37°C until the evolution of free sialic acid was complete (usually 2.0—3.0 h). Under these conditions no loss of carboxypeptidase N activity occurred, in contrast to the considerable loss of activity which developed when incubation was performed at pH 5.0.

Assays. Samples for carboxypeptidase N determination were made up to 0.5 mM with $CoCl_2$ and incubated at 31°C for 3 h. 0.1 ml enzyme solution was then incubated with 0.1 ml 20.0 mM hippuryl-L-lysine in 0.2 M potassium phosphate buffer pH 7.0 at 37°C for 30 min and the reaction terminated by addition of 0.2 ml 1.0 M HCl. Hippuric acid was extracted with 1.5 ml ethyl-acetate [10,11] and 1.0 ml of the extract evaporated to dryness at 120°C. The dried material was dissolved in 3.0 ml of 1 M NaCl and absorbance determined at 228 nm versus appropriate blanks consisting of enzyme-substrate solutions to which HCl had been added at zero time. Protein was determined by absorbance at 280 nm, by the method of Lowry et al. [12]. Sialic acid was determined by the thiobarbituric acid assay of Warren [13]. One unit of enzyme activity was taken as equivalent to the evolution of 1 μmol of product per minute.

Partial purification of carboxypeptidase N. Blood was collected from healthy donors in glass tubes and allowed to clot at 4°C. 10 ml serum was diluted to 50 ml with distilled H_2O to a conductivity of 2.5 $m\Omega^{-1}$/cm and held at 4°C overnight [4]. Euglobulin was removed by centrifugation. The supernatant was

applied to a cellulose column and eluted with 100 ml 0.02 M potassium phosphate buffer pH 7.0 and then with a gradient containing 0 to 0.3 M NaCl in buffer. Fractions containing carboxypeptidase N were pooled, concentrated by Amicon pressure filtration with a 10 μm membrane to 50 ml and dialyzed versus 0.07 M NaCl and 0.1 mM $CoCl_2$ in 0.02 M potassium phosphate pH 7.0. The dialyzed enzyme was then reapplied to a TEAE-cellulose column and eluted with a NaCl gradient (0.07—0.3 M) in 0.02 M potassium phosphate buffer pH 7.0. Fractions containing enzyme were pooled, concentrated to 25.0 ml as above and dialyzed against 0.1 mM $CoCl_2$ in 0.02 M potassium phosphate pH 7.0 prior to isoelectric focusing.

Results

Fig. 1 illustrates the results obtained when carboxypeptidase N was exposed to isoelectric focusing for 24 to 72 h. At 24 and 42 h two major charged species were apparent whereas at 72 h loss of anodic activity was prominent with the appearance of several activity peaks in the higher pH range. The results suggest that the two peaks obtained at 24 h (Fig. 1a) represent incompletely focused material since there was considerable migration of material from the cathodic to the anodic peak during the ensuing 16 h (Fig. 1b). The isoelectric points at maximum peak activity were quite similar at these two time periods however, the most anodic material having an isoelectric point of pH 3.8 while the cathodic material had an isoelectric point of pH 4.3. The pattern shown in Fig. 1b was relatively stable for several samples between 40 and 50 h suggesting that focusing was relatively complete at this time. Thus carboxypeptidase N appears to consist of at least two charged species in serum. It is clear from Fig. 1b however that minor species with isoelectric points of pH 4.6 and 4.9 were also present. The results obtained at 72 h can only be explained on the

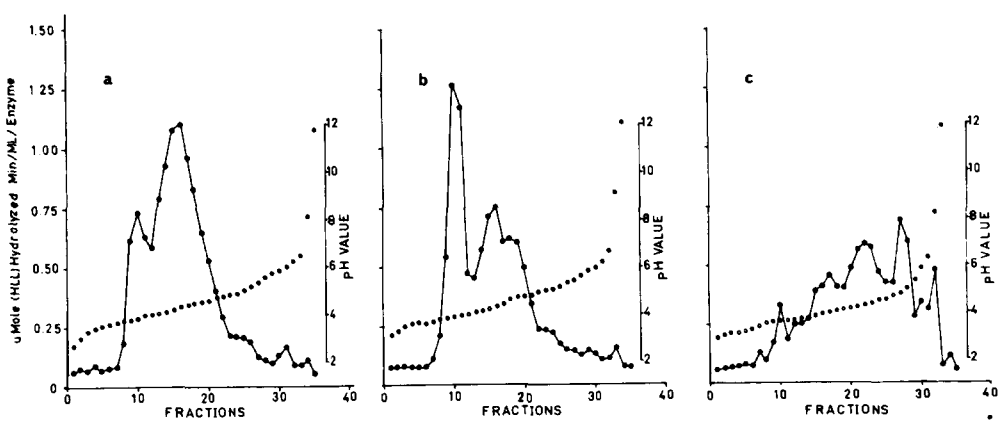

Fig. 1. Isoelectric focusing of carboxypeptidase N. Focusing experiments performed with 110 ml column in 5—50% w/v sucrose density gradient containing 1% w/v ampholine, pH 2.5—7.0. ●———●, hydrolysis of substrate hippuryl-L-lysine (HLL); ○———○, pH ampholine carrier ampholite gradient. (a) 24 h, (b) 42 h, (c) 72 h.

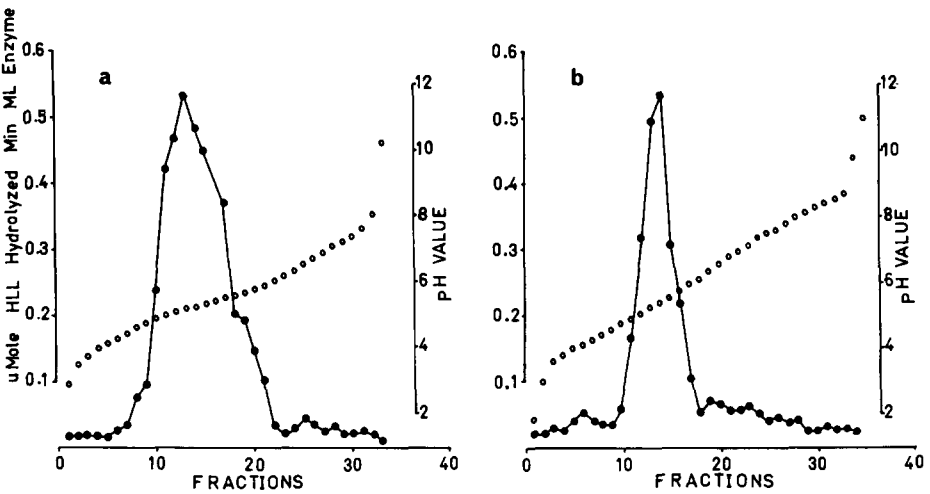

Fig. 2. Isoelectric focusing of carboxypeptidase N after incubation with neuraminidase. Focusing experiments performed in 5—50% w/v sucrose density gradient for 42 h. (a) Focusing in 1% w/v ampholine gradient pH 4.0—8.0. (b) Refocusing in 1% w/v ampholine gradient pH 3.0—10.0. ●———●, hydrolysis of substrate hippuryl-L-lysine (HLL); ○———○, pH ampholine carrier ampholite gradient.

basis of a major loss of charge from the anodic components present at 42 h resulting in the dominance of species with isoelectric points of pH 4.0, 4.3, and 4.8. Recovery of carboxypeptidase N activity was in excess of 80% at the early time but much reduced at 72 h. Subsequent focusing experiments were therefore performed over a period of 40 to 50 h.

Loss of anodic charge during focusing suggested the possibility to us of

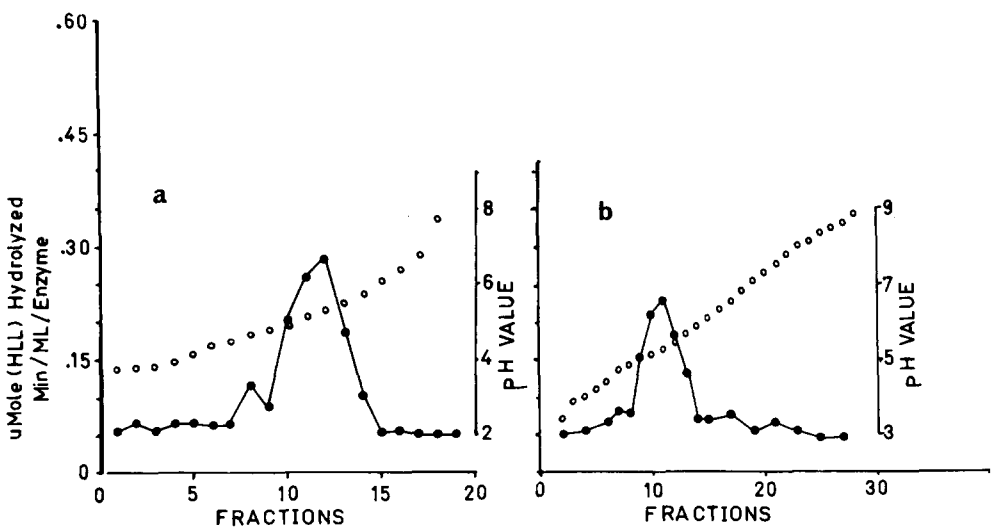

Fig. 3. Refocusing of material from two activity peaks obtained at 42 h as in Fig. 1b following desialation. (a) Refocusing of anodic peak in pH gradient 3.0—8.0, (b) Refocusing of cathodic peak in pH gradient 3.0—10.0. ●———●, hydrolysis of substrate hippuryl-L-lysine (HLL); ○———○, pH ampholine carrier ampholite gradient.

progressive hydrolysis of an acid labile anion during focusing. Since sialic acid was a prime candidate, the enzyme was subsequently desialated as described in methods. Identical results were obtained with two sets of neuraminidase, one rendered protease free and provided as a gift. As shown in Fig. 2a desialation of material provided by TEAE-cellulose chromatography resulted in a single homogeneous peak material with an isoelectric point of 5.2, without appreciable loss of enzyme activity since the recovery from the column was approximately 84% of that applied. Refocusing of this material resulted again in the appearance of a single dominant species with an isoelectric point of 5.4. With refocusing of the desialated enzyme recoveries dropped somewhat ranging from 30 to 70%. To determine whether both major peaks of the sialo protein gave rise to the same asialo product, the two major fractions from 42 h focusing runs were refocused as shown in Fig. 3. Both fractions gave rise to carboxypeptidase N peaks with an p*I* range of pH 5.0 to 5.6 and a p*I* of pH 5.3 at maximum peak height.

Discussion

Porcine serum carboxypeptidase N has recently been shown to be a glycoprotein by virtue of its staining with the periodic acid-Schiff reagent and binding by concanavalin A [14]. In the present study we have demonstrated a dependence of the isoelectric focusing pattern upon sialic acid which strongly implies that the human serum enzyme is also a glycoprotein. Interestingly desialation appears to have had little influence upon catalytic activity since recovery of activity was nearly complete after removal of sialic acid residues. It was noted however that loss of activity occurred when we attempted to refocus the desialated species indicating that the sialic acid residues may be of importance in stabilizing the catalytic form of the enzyme.

Prolonged isoelectric focusing resulted in loss of most of the highly sialated enzyme activity presumably because of hydrolysis of sialic acid groups in the lower pH range of the gradients. Since at least four species appeared on prolonged focusing each with a p*I* lower than that of the completely desialated enzyme it seems likely that the fully sialated species contains multiple sialic acid residues which are susceptible to sequential cleavage.

Although new species were obviously created during focusing, the earliest focusing periods invariably demonstrated two major activity peaks implying strongly that the circulating enzyme probably consists of two separable charged species. Further work will be required to determine the origin of these apparent isoenzymes. Oshima et al. [15] have argued that much of the circulating enzyme activity is derived from liver but the possibility of contributions from other organs has not been excluded. Organ specific isoenzymes of alkaline phosphatase are separable on the basis of their response to neuraminidase [16] and the same situation may hold for carboxypeptidase N. The apparent homogeneity of the desialated enzyme demonstrated in this report suggests that it is relatively unlikely that carboxypeptidase N isoenzymes are coded for by separate genes, although it is difficult to be conclusive on this point on the basis of a single analytical technique. Post translation modification either through the impact of circulating neuraminidase or the variable efficiency of

sialyltransferases during synthesis must also be considered as a possible mechanism which might produce isoenzymes of differing sialic acid composition.

Removal of sialic acid from circulating glycoproteins is a major determinant of their rates of clearance from plasma [17,18]. It is tempting to speculate upon the role of tissue and plasma neuraminidases as possible determinants of the level of serum carboxypeptidase N activity. Loss of sialic acid might lead to rapid loss of enzyme and thereby could provide a sensitive method for controlling the degradation rate of kinins and products of the complement system. Since carboxypeptidase N retains its activity following desialation the enzyme might also provide a convenient molecule for studies of hepatic clearance mechanisms involving glycoproteins.

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