BBA Report

BBA 31101

Isoenzymes of carbonic anhydrase I from primate red blood cells

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(Received February 22nd, 1971)

SUMMARY

The electrophoretic patterns of the multiple forms of carbonic anhydrase I were examined from the red cells of two species of macaque monkeys and orangutan. In the rhesus macaque, *Macaca mulatta*, it was possible to generate both anodal and cathodal electrophoretic components when a single component was isolated and electrophoresed again. These findings suggest that the alternate forms of carbonic anhydrase I are conformational isoenzymes rather than the result of such processes as binding, deamination, and proteolytic action.

Two genetically different molecular forms of carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1), designated carbonic anhydrase I and carbonic anhydrase II (or B and C, respectively), have been identified from the red cells of man and a number of primate species[1-4]. A third electrophoretic component in human and other mammalian red cells has been designated carbonic anhydrase I (+1) (*cf.* ref. 5), and by others as carbonic anhydrase A (*cf.* ref. 6). All three of the human carbonic anhydrases are single polypeptide chains of approximately 265 amino acid residues with molecular weights of about 30 000[2,7,8].

The specific CO_2 hydrase activity of human carbonic anhydrase I (+1) is similar to carbonic anhydrase I as is its amino acid composition, tryptic peptide pattern, and immunochemical specificity (*cf.* refs. 9 and 6); however, the tertiary configuration of these two forms of carbonic anhydrase I appears to differ[6].

The changes in electrophoretic mobility seen in the genetic variants of the carbonic anhydrase I of man and other primates are reflected in the mobility of their respective carbonic anhydrase I (+1)'s; *i.e.*, electrophoretic variation in carbonic anhydrase I (+1) segregates with carbonic anhydrase I[10,11]. These findings indicate that this minor

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component of carbonic anhydrase I is not determined by a genetic locus distinct from carbonic anhydrase I, but represents an alternate molecular form of carbonic anhydrase I.

The study reported here was designed to examine some of the properties of the multiple forms of carbonic anhydrase I from primate red cells. We believe that the observations on these multiple forms provide significant support for the concept of non-genetic, interchangeable, conformational modifications of the carbonic anhydrase I molecule, which have been termed conformational isoenzymes or conformational isomers (conformers)[12,14].

The principal species used for this study was the rhesus macaque (*Macaca mulatta*) since its red cells contain a relatively large amount of carbonic anhydrase I of high specific esterase activity, and a large number of easily detected electrophoretic forms[5,15]. The starch gel electrophoretic technique and staining procedure for carbonic anhydrases have been outlined in detail elsewhere[5]. Zones of carbonic anhydrase activity on the gel were detected by staining for esterase activity using both α- and β-naphthylacetate as substrates.

The electrophoretic pattern of rhesus red cell carbonic anhydrase I on starch gel, stained for both esterase activity and protein (Nigrosin stain) is shown in Fig.1. All bands of esterase activity shown here were abolished when the specific carbonic anhydrase inhibitor, acetazolamide, was added to the substrate–dye mixture, confirming that all were carbonic anhydrase. 4 bands of activity were clearly evident in a hemolysate, while at least 6 additional bands of carbonic anhydrase I were seen in partially purified and concentrated extracts. Fig.1 also illustrates the fact that electrophoretic mobility of these forms is remarkably stable during storage at $-20°$. An occasional additional band has been noted as illustrated in channel 4 of the esterase pattern.

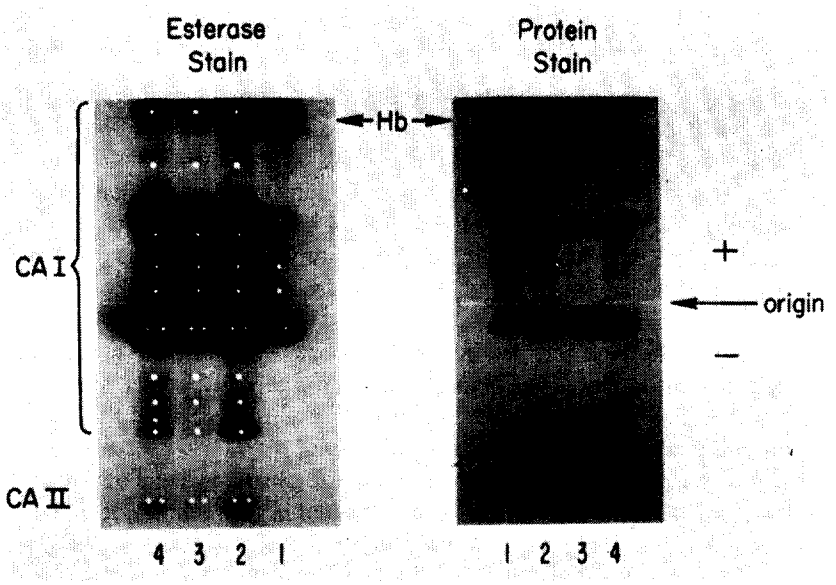

Fig.1. Effect of storage on the electrophoretic patterns of rhesus macaque red cell carbonic anhydrases. Hemolysate (1); a concentrated chloroform–ethanol extract partially purified on a DEAE-cellulose column and stored at $-20°$ for varying times, fresh (2); 9 months (3); 7 months (4). Double white dots and single white dots, respectively, identify major and minor forms of carbonic anhydrase (CA).

Individual bands were isolated by cutting strips from the gel and removing the protein by electrodialysis in the borate buffer (pH 8.6) which was used for preparing the starch gel[16]. Re-electrophoresis of the isolated proportions gave the esterase and protein pattern shown in Fig.2. It appears that the isolated forms of carbonic anhydrase I generate other anodal and cathodal electrophoretic forms of the enzyme which have mobilities comparable to those in the original preparation. By testing the immunochemical properties of each solution in a double immunodiffusion system on agar coated slides[17] the protein from all except the two most cathodal bands cross reacted with antiserum made against human red cell carbonic anhydrase I. Protein from the two cathodal bands (channels 7 and 8, Fig.2) cross reacted with antiserum against the other isoenzyme of carbonic anhydrase, carbonic anhydrase II. The antisera were prepared in rabbits as described earlier[3].

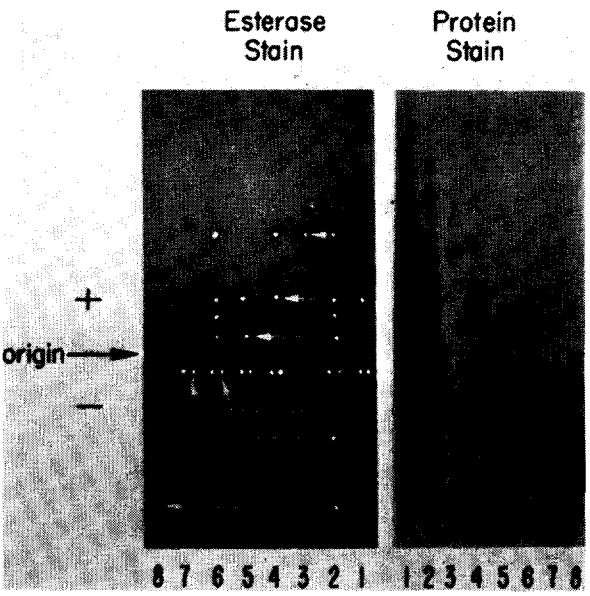

Fig.2. Re-electrophoresis of various electrophoretic forms of rhesus red cell carbonic anhydrase I isolated from starch gel. Hemolysate (1); chloroform–ethanol extract of hemolysate partially purified on a DEAE-cellulose column and concentrated (2). In channels 3–8 arrows indicate individual zones of esterase activity from channel 2 which had been isolated from starch gel and re-electrophoresed, showing generation of other forms.

Starch gel electrophoresis separates molecules on the basis of molecular charge and size. Discrimination on the basis of charge alone was achieved by subjecting a partially purified preparation of rhesus hemolysate to isoelectric focusing in a linear sucrose gradient and using LKB Ampholine carrier ampholytes with a pH range of 5–8. After equilibration was achieved, fractions were collected and both pH and absorbance at 280 nm were determined (Fig.3). The fractions were then pooled into 6 groups each of which was dialyzed against 3 mM phosphate buffer (pH 7.0) to free them of Ampholine, concentrated in Visking casing, and subjected to starch gel electrophoresis which produced esterase and protein patterns as shown in Fig.4. Clearly, isoelectric focusing in a fluid medium distributes the carbonic anhydrase I forms along the pH gradient.

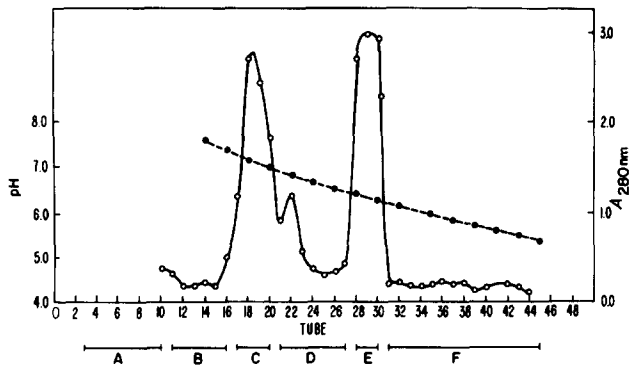

Fig.3. Elution pattern of fractions collected from an electrofocusing column. The starting enzyme preparation was 26 mg of protein in a chloroform–ethanol extract of hemolysate from a single rhesus macaque and partially purified on a DEAE-cellulose column. After measurement of pH and absorbance, fractions were pooled as indicated by letters A to F.

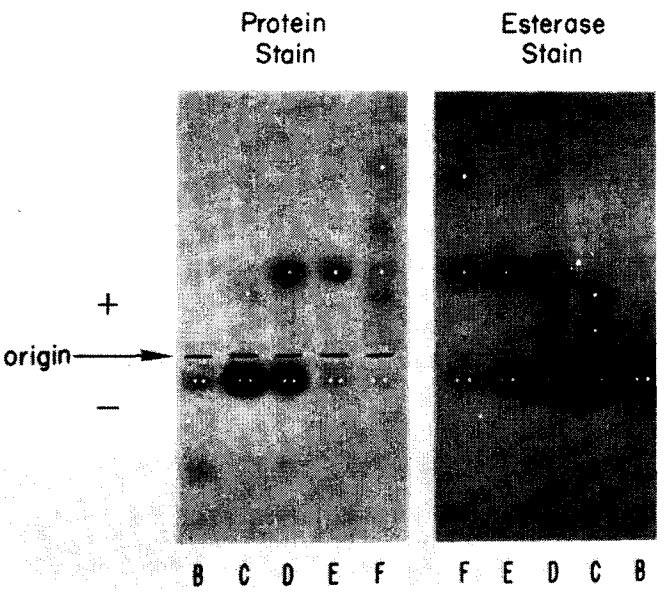

Fig.4. Starch gel esterase zymogram of pooled fractions from an electrofocusing column. Lettered channels correspond to the pooled fractions in Fig.3. The top half of the gel was stained for esterase activity and the white dots identify zones of esterase activity which were sensitive to acetazolamide. Double dots label the position occupied by the major component of carbonic anhydrases I and II (CA I and CA II). Before staining for protein the bottom half of the sliced gel was covered with bromthymol blue and tested for zones of hydrase activity by flushing the surface with CO_2. The protein bands identified by white dots possessed hydrase activity.

In Fig.5 is shown the electrophoretic pattern of a partially purified preparation of orangutan (*Pongo pygmaeus*) hemolysate in concentrations from 5 to 80 mg/ml of protein, as measured by the Lowry method[18], indicating that the number of visible forms is a function of enzyme concentration. Also, at 80 mg/ml, the number of visible forms of carbonic anhydrase I is similar to that in hemolysates from rhesus macaques (Fig.1)

containing 2.3 mg/ml as measured by a sensitive radioimmunoassay[19]. The relative distribution of carbonic anhydrase I forms along the electrical gradient is different for these two species. In contrast, among closely related species of macaques there is similarity in number of electrophoretic forms of red cell carbonic anhydrase I at equivalent protein concentration and in their relative electrophoretic mobilities[11]. These observations on differences among species support the view that the number and mobilities of multiple forms of carbonic anhydrase I in a given species is a function of the particular amino acid sequence of its carbonic anhydrase I.

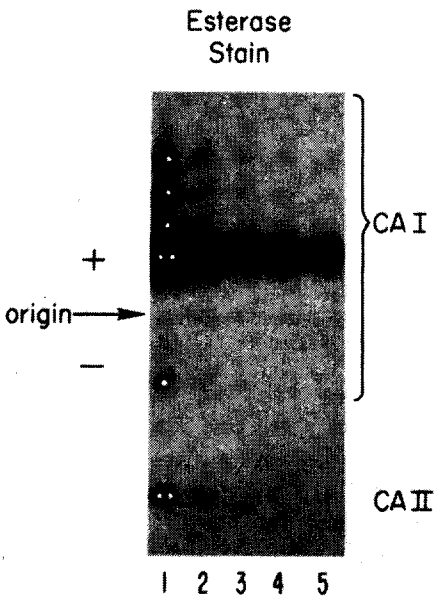

Fig.5. Starch gel esterase zymogram of chloroform–ethanol extract of orangutan hemolysate partially purified on a DEAE-cellulose column. 25 μl were inserted into each well in the starch gel. Protein concentrations for channels 1 to 5 were 80, 40, 20, 10 and 5 mg/ml, respectively.

Multiple electrophoretic forms of carbonic anhydrase I shown here are not to be confused with genetic variants. This distinction was clearly made for human carbonic anhydrase I[10], and is shown in Fig.6 for genetic variants of carbonic anhydrase I in the pig-tailed macaque (*Macaca nemestrina*). These variants were shown by pedigree studies to be alleles at a single genetic locus[11]. It is clear that each genetic variant has a unique minor electrophoretic form associated with it.

Relative proportions for the multiple forms of a given carbonic anhydrase molecule have not been investigated in detail. It is significant to note, however, that an electrophoretic variant of red cell carbonic anhydrase I in the Japanese macaque (*Macaca fuscata*) presents as two major components of about equal amounts, in addition to other minor forms[11]. In other genetic variants among the various primates a single major component occurs and the largest minor component constitutes about 6% of the total red cell carbonic anhydrase I.

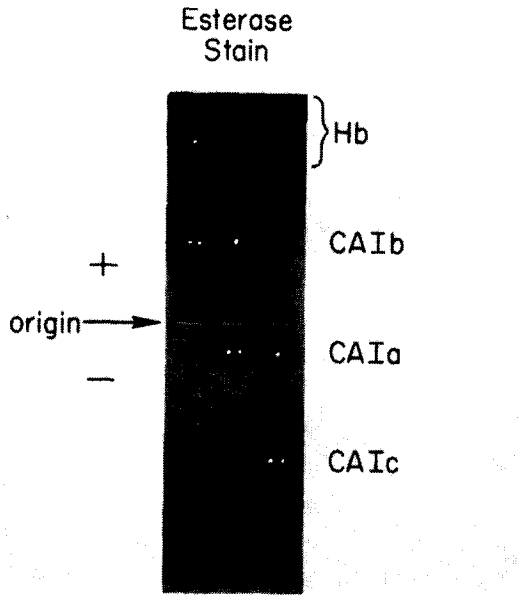

Fig.6. Three genetic variants of carbonic anhydrase I (CA I) in red cell hemolysates of the pig-tailed macaque. The single white dot indicates the (+1) form of each variant.

Several authors have suggested that the primary structure of a protein can permit a variety of conformational arrangements which alter net surface charge[12–14]. This is also indirectly supported by studies on carbonic anhydrase I isoenzymes of man and rhesus macaque[20,21,4]. Conversion from one electrophoretic form to another has been accomplished by changing pH or by placing the enzyme in urea. Funakoshi and Deutsch[20,21] generated new anodal forms from single isolated forms of human red cell carbonic anhydrase I by adjusting the enzyme solution to about pH 12 and attributed the new forms to a loss of amide groups. The data shown in Fig.2 demonstrate that under the relatively mild conditions of pH 8.6 a given isolated form can generate forms within the same sample which migrate toward both the anode and cathode. It is difficult to reconcile this observation with the concept of amide loss as a sufficient basis for carbonic anhydrase isoenzyme formation. Another potential interpretation of these data could be based on the presupposition that aggregation of carbonic anhydrase molecules occurs. This is rendered improbable by the observation that the I and I (+1) forms of human red cell carbonic anhydrase have essentially identical molecular weights[2,7,8].

The results of this study indicate that differences in multiplicity of minor electrophoretic forms of carbonic anhydrase I, their relative electrophoretic mobilities and the relative proportions in each form are associated with phylogenetic and genetic differences. This supports the hypothesis that these isoenzymes are a function of the primary enzyme structure. Whether these isoenzymes occur *in vivo* remains uncertain. Artifactual elements have not been entirely excluded; however, these are becoming increasingly unlikely since multiplicity of forms has now been shown in the presence of a variety of buffers and electrophoretic supporting media.

We thank Dr. Robert J. Tanis for electrofocusing the rhesus macaque carbonic anhydrase. This project was supported by grants from the U.S. Public Health Service (5-T01-GM-71 and GM-15419).

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