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Characterization of a Mutant Human Erythrocyte Carbonic Anhydrase: Carbonic Anhydrase Ic_{Guam}[1]

The Amino Acid Substitution and Carboxylesterase and Hydratase Activities

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Evidence is presented which indicates that an arginine residue has substituted for a glycine residue in the abnormal human erythrocyte carbonic anhydrase, CA Ic Guam. The ratio of the amount of mutant to normal enzyme isolated from hemolyzates of individuals heterozygous for the variant averaged 0.56:1. No significant differences were observed between the specific carboxylesterase activities or specific CO_2 hydratase activities of the normal and variant carbonic anhydrases.

Two distinct molecular forms of red cell carbonic anhydrase from a number of mammalian species have been described (1–3). In man, the two forms have been designated as carbonic anhydrase I (CA I) and carbonic anhydrase II (CA II) or carbonic anhydrases B and C, respectively (1, 4). Both of these forms appear to be composed of single polypeptide chains (M.W. $\sim$ 30,000) with one zinc atom and one cysteine residue per enzyme molecule; however, they differ in such properties as relative amounts synthesized, amino acid composition, and specific enzyme activities (cf. 4–6).

Several presumed or proved, genetically determined, electrophoretic variants in both of these carbonic anhydrase forms from man and nonhuman primate species have recently been reported (1). The present communication further characterizes one of these abnormal carbonic anhydrases; an inherited variant of CA I, termed CA Ic_{Guam}, which was discovered in a Micronesian population inhabiting the Mariana Islands of Guam and Saipan (7). Through the kind cooperation of Mr. C. C. Plato and Dr. G.-M. Chen and their colleagues of the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service, a sufficient quantity of blood was collected from several individuals heterozygous for the CA Ic variant to permit a comparative chemical study of the primary structure of the normal and variant enzymes as well as their CO_2 hydratase and carboxylesterase (8) activities.

Throughout this report, the normal form of CA I will be referred to as CA Ia and the variant form as CA Ic.

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EXPERIMENTAL PROCEDURE

Preparation of the carbonic anhydrases. Whole blood was mixed with 0.17 volume of acid-citrate-dextrose, packed in ice, and shipped in insulated containers. Twenty-five ml of packed red blood cells was washed 3 times with isotonic NaCl, and the hemolyzates were prepared by adding one volume of distilled water to one volume of packed cells. Enzyme purification was initiated by a modification of the chloroform-ethanol extraction procedure (9). One volume of 40% ethanol and 0.5 volume of chloroform were added at the same time to one volume of hemolyzate, which was extracted by vigorous shaking for 5 minutes; the mixture was then allowed to stand for 30 minutes. After centrifugation at 2000 rpm for 15 minutes, the ethanol layer was removed. The mass of cellular debris and denatured protein was extracted again

with 50 ml of 20% ethanol in a Waring Blendor for 45 seconds and centrifuged as before. The 2 ethanol extracts were combined, concentrated in the cold by ultrafiltration in Visking casing to a volume of about 5 ml, and dialyzed against 3×10^{-3} M phosphate buffer, pH 7.0, for 24 hours. The carbonic anhydrases were further purified by absorption of contaminating proteins on a diethylaminoethyl (DEAE) cellulose column (0.89 meq per gram, 1 × 15 cm) which had been equilibrated with 3×10^{-3} M phosphate buffer, pH 7.0. Figure 1 shows the electrophoretic patterns of the proteins after the respective chloroform-ethanol and DEAE purification steps. Final purification of the enzyme forms was achieved by preparative starch-gel electrophoresis. The protein solution was concentrated in the cold to a volume of 0.3–0.5 ml by ultrafiltration in collodion bags, porosity 5 mμ (Schleicher and Schuell Co.), and subjected to starch-gel electrophoresis in 0.02 M borate/NaOH buffer, pH 8.75, as previously described (10). The position of the enzymes on the gel was determined by cutting a thin test strip along the edge of the outer channel of the gel slab and staining for protein and esterase activity as previously reported (7). The areas adjacent to the stained zones were cut from the gel, and the enzymes were removed by electrophoresis in 0.02 M borate/NaOH buffer, pH 8.75, at 2° for 18 hours essentially as described by Koen and Shaw (11). Finally, the solutions containing the purified enzymes were centrifuged for 20 minutes at 34,000*g*, dialyzed against distilled water, and concentrated to the desired volume in collodion bags.

Carboxylesterase assays. The esterase activities of the carbonic anhydrases were determined by following the rate of β-naphthol formation from β-naphthyl acetate and *p*-nitrophenol formation from *p*-nitrophenyl acetate.

To test for the activity toward β-naphthyl acetate, the enzyme preparation was incubated for 20 minutes at 37° in a total volume of 2 ml containing 1.25×10^{-3} M recrystallized β-naphthyl acetate (K and K Laboratories, Inc.) 0.05 M Tris-HCl buffer (pH 8.5), 2% *p*-dioxane (Eastman Organic Chemicals), and 0.5% polyoxyethylene lauryl ether (Brij 35, Atlas Chemical Industries, Inc.). The reaction was stopped by adding 1 ml of 4×10^{-3} M acetazolamide (Diamox, Lederle Laboratories). One ml of 0.2% diazotized *p*-chloro-*o*-toluidine (Fast Red TRN, Dajac Laboratories) was then added to the mixture, and the color (β-naphthol-azo dye complex) was allowed to develop at room temperature for 45 minutes. A 0.5-ml aliquot of the colored solution was diluted with 1.5 ml of 95% ethanol, centrifuged at 2000 rpm for 10 minutes, and the absorbancy was measured in a spectrophotometer at 490 mμ against a reagent blank to which 4×10^{-3} M acetazolamide had been added at zero time.

The activity toward *p*-nitrophenyl acetate was measured by incubating the enzyme for 20 minutes at 37° in 2.5 ml of 2.4×10^{-3} M phosphate buffer (pH 7.05), containing 8×10^{-4} M *p*-nitrophenyl acetate (Sigma Chemical Co.), and 0.06% polyoxyethylene lauryl ether. The enzyme activity was stopped by the addition of 1 ml of 4×10^{-3} M acetazolamide in 3×10^{-3} M phosphate buffer, pH 7.05, and the absorbancy of the solution was read immediately in a spectrophotometer at 400 mμ against a reagent blank to which acetazolamide (4×10^{-3} M) had been added at zero time.

The relationship of absorbancy to the concentration of β-naphthol and *p*-nitrophenol standards followed Beer's law within the concentration range of *p*-nitrophenyl acetate and β-naphthyl acetate that was used.

CO_2 hydratase assay. The hydration of CO_2 was measured by the method of Nyman (12) where the rate of change in the ultraviolet absorbancy of Veronal buffer, which accompanies the base to acid shift, was followed in a Gilford (model 2000) recording spectrophotometric system with thermospacer and temperature-measuring accessories. The enzyme, in 2 ml of 0.02 M Veronal-HCl buffer, pH 8.8, was equilibrated to 0.5 ± 0.02° in the cuvette housing, maintained at that temperature by a Haake Constant Temperature Circulator (model KT-63). The reaction was initiated by the addition of 1 ml of ice-cold, CO_2-saturated deionized water, and the decrease in absorption at 276 mμ during a 30-second interval was used as the index of hydratase activity.

Figure 2 shows the linear relationships between the amount of CA Ia and its esterase and CO_2 hydratase activities.

Proteins were determined by the method of Lowry *et al.* (13) with bovine albumin as the standard.

Peptide and amino acid analysis. The basic procedures described by Katz *et al.* (14) were modified for peptide mapping. About 20 mg of enzyme was suspended in 2 ml of 0.2 M ammonium carbonate buffer, pH 8.5, and denatured by heating at 95° for 3 minutes. The denatured protein was then washed twice with 2 ml of the ammonium carbonate buffer, resuspended in 1 ml of the same buffer, and digested with 2% (by weight) trypsin (2× crystallized, Worthington Biochemicals Corp.) for 90 minutes at 37°. The digest was then centrifuged for 10 minutes at 3000 rpm to separate the undigested "core" which represented about 35–40%[2] of the total protein. The "core" was washed twice

[2] Determined from total amino acid composition of the tryptic and chymotryptic digests.

with ammonium carbonate buffer and digested in 0.5 ml of the buffer with 1% chymotrypsin (3× crystallized, Worthington) for 90 minutes at 37°. About 2.5-mg portions of the tryptic or chymotryptic digests were applied to each sheet of Whatman 3MM filter paper. Descending chromatography was carried out in butanol-pyridine-acetic acid-water (15:10:13:12) and electrophoresis in pyridine-acetic acid-water (25:1:250), pH 6.5, at 2000 V (160–180 mA) for 85 minutes. After drying, the filter paper sheets were sprayed with with 0.2% ninhydrin in 95% ethanol. The ninhydrin-free peptides to be anayzed were separated by high voltage electrophoresis for 2 hours. The peptides were located with ninhydrin on guide strips, eluted with 3 times glass-distilled 6 N HCl and hydrolyzed for 20 hours at 105° in evacuated tubes. The hydrolyzates were evaporated to dryness in an evacuated desiccator over KOH pellets and phosphorous pentoxide and analyzed on a Technicon amino acid analyzer. No corrections were made for the destruction of the labile amino acids. Ninhydrin-negative peptides were detected by the method of Rydon and Smith (15). The average residue values were estimated from the yields of amino acids stable to acid hydrolysis.

RESULTS AND DISCUSSION

Relative synthesis of normal and variant carbonic anhydrases. The isolation of CA Ia and CA Ic from 4 different heterozygous samples gave the following yields from 1 ml of packed red cells: CA Ia (1.1, 1.1, 0.9, 0.8 mg) and CA Ic (0.6, 0.6, 0.5, 0.5 mg). Thus, the average ratio of mutant to normal enzyme is 0.56:1. That this disproportional synthesis of 2 allelic products is not the result of the purification procedure is indicated by the fact that CA Ia and CA Ic isolated from untreated hemolyzates and chloroform-ethanol extracts by preparative starch-gel electrophoresis gave essentially similar ratios of mutant to normal enzymes of 0.62:1 and 0.60:1, respectively.

On the basis of an analogy with the electrophoretic pattern of CA Ia and its minor component, CA Ia(+1) [identical to CA A of Rickli *et al.* (4)], it is probable that the presumed minor component of CA Ic, CA Ic(+1), overlaps CA Ia in the pattern shown in Fig. 1. However, since the ratio of CA I to CA I(+1) from normal hemolyzates is about 15:1, this would mean that CA Ia extracted from heterozygous hemolyzates is contaminated with CA Ic(+1) only to the extent

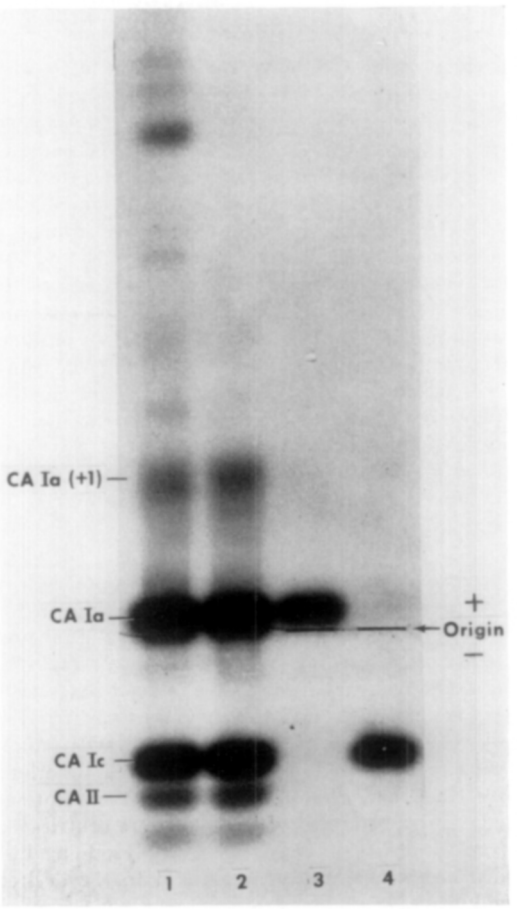

FIG. 1. Protein patterns after starch-gel electrophoresis at different stages of purification. Chloroform-ethanol extract, 1; DEAE-cellulose absorption, 2; preparative electrophoresis of CA Ia, 3; and preparative electrophoresis of CA Ic, 4. Nigrosin was used for the protein stain and electrophoresis was carried out as previously reported (10). CA Ia probably also includes some CA Ic(+1); see text.

of about 6% of CA Ic, or approximately 0.03 mg.

Quantitative determinations of CA I from 4 normal hemolyzates ranged from 1.5 to 1.7 mg (average 1.6 mg) from 1 ml of packed cells. Since the average total CA I from the heterozygous hemolyzates averaged 1.5 mg per milliliter of packed cells, it appears that approximately the same total amount of CA I is being synthesized in both normal and heterozygous erythrocytes.

The synthesis of the other form of carbonic

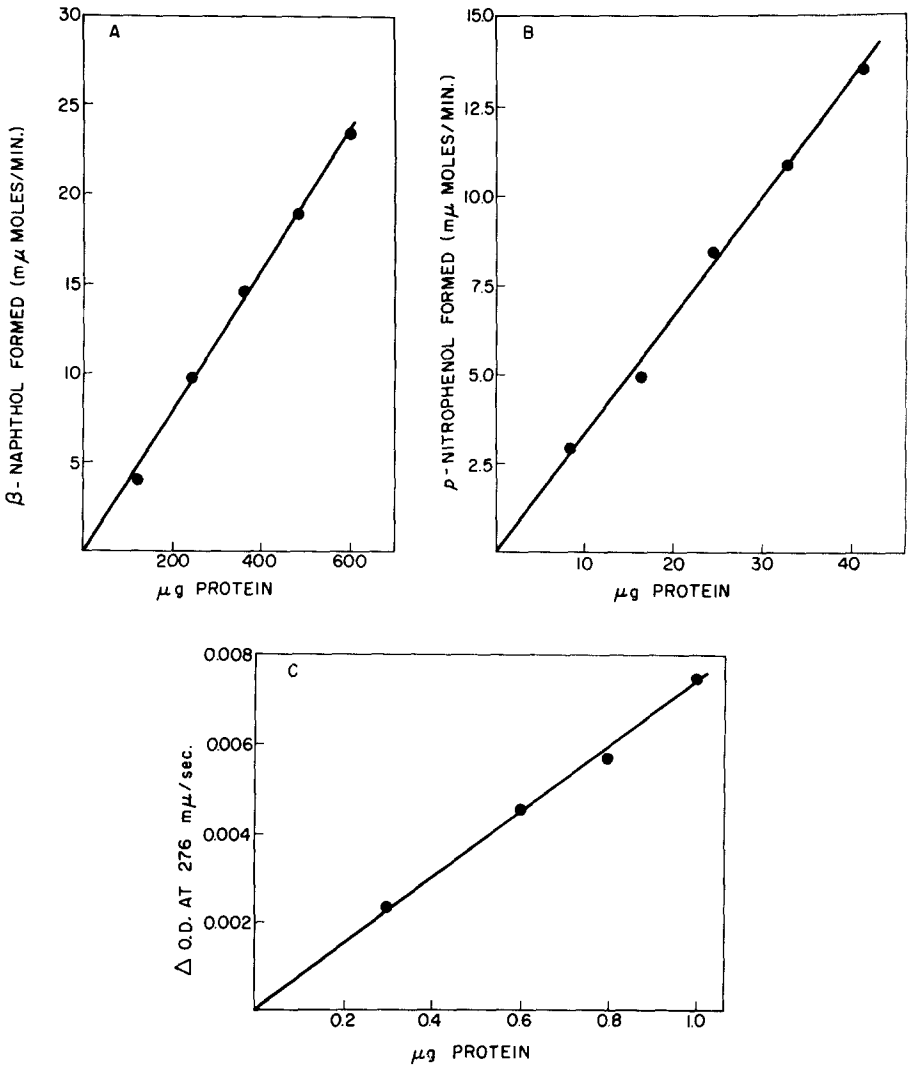

FIG. 2. Effect of the concentration of carbonic anhydrase Ia on the rate of (A) β-naphthol formed from β-naphthyl acetate; (B) *p*-nitrophenol formed from *p*-nitrophenyl acetate; and (C) CO_2 hydration as determined by the change in absorbance of Veronal buffer. See text for details.

anhydrase, CA II, does not appear to be altered in the variant hemolyzate, since an average of 0.06 and 0.07 mg of CA II per 1 ml of packed cells was extracted from the normal and variant hemolyzates, respectively.

Compartive enzyme activities. The carboxylesterase activities and CO_2 hydratase activities of the normal and variant carbonic anhydrases are listed in Table I. No deviation from normal values is apparent in the enzyme activities of the CA Ic variant enzyme. Also, the activities of CA II do not differ between the normal and variant hemolyzates.

Comparison of tryptic peptide patterns. Examination of the tryptic peptide maps of CA Ia and CA Ic reveals the absence of peptide T5 in CA Ic and the presence of two new peptides T5c and T1a (Fig. 3). An increase in the staining intensity of T3 in the CA Ic pattern is also apparent. It was not possible to make a very meaningful comparison of the chymotryptic patterns because of the quantitative variation in some of the peptides; however, no obvious qualitative

peptide alterations were ever observed under optimal staining conditions. Treatment of the separated tryptic peptides of CA Ia with chlorine (15) failed to show any additional peptides not observed by the ninhydrin method.

TABLE I

COMPARISONS OF THE CARBOXYLESTERASE AND CO_2 HYDRATASE ACTIVITIES OF ERYTHROCYTE CARBONIC ANHYDRASES FROM NORMAL AND VARIANT HEMOLYZATES

Values represent the average of 3 separate assays.

Molecular form	Carboxylesterase activity		CO_2 Hydratase activity (units[c]/mg)
	β-Naphthyl acetate (units[a]/mg)	p-Nitrophenyl acetate (units[b]/mg)	
	Variant hemolyzate		
CA Ia	0.044	0.32	7.61
CA Ic	0.049	0.35	7.88
CA II	0.042	1.61	25.99
	Normal hemolyzate		
CA Ia	0.045	0.33	7.43
CA II	0.040	1.60	26.58

[a] μMoles β-naphthol formed/minute.
[b] μMoles p-nitrophenol formed/minute.
[c] ΔOD at 276 mμ/second.

The amino acid substitution. Table II lists the amino acid composition of the CA Ia tryptic peptides T1, T3, T4, and T5 and CA Ic peptides T1, T1a, T3, T4, and T5c. The change in the composition of T5 from Gly-Arg (yellow ninhydrin reaction) to T5c, which is composed entirely of arginine (blue ninhydrin reaction), indicates that glycine in T5 has been substituted for an arginine residue. That T5c is not free arginine, but probably the dipeptide Arg-Arg, is based on the evidence that T3 is free arginine. Not only does an arginine standard migrate to the T3 position but, as shown in Table II, when T3 is eluted with 1 N acetic acid and analyzed without acid hydrolysis treatment, the yield of free argining is comparable with that from an acid-hydrolyzed sample of T3. The increased yield (Table II) and staining intensity (Fig. 3) of T3 from CA Ic could be accounted for by the tryptic cleavage of some free arginine from T5c (Arg-Arg). Peptide T1a, which is composed of equal amounts of lysine and arginine, may represent the consequence of a secondary effect

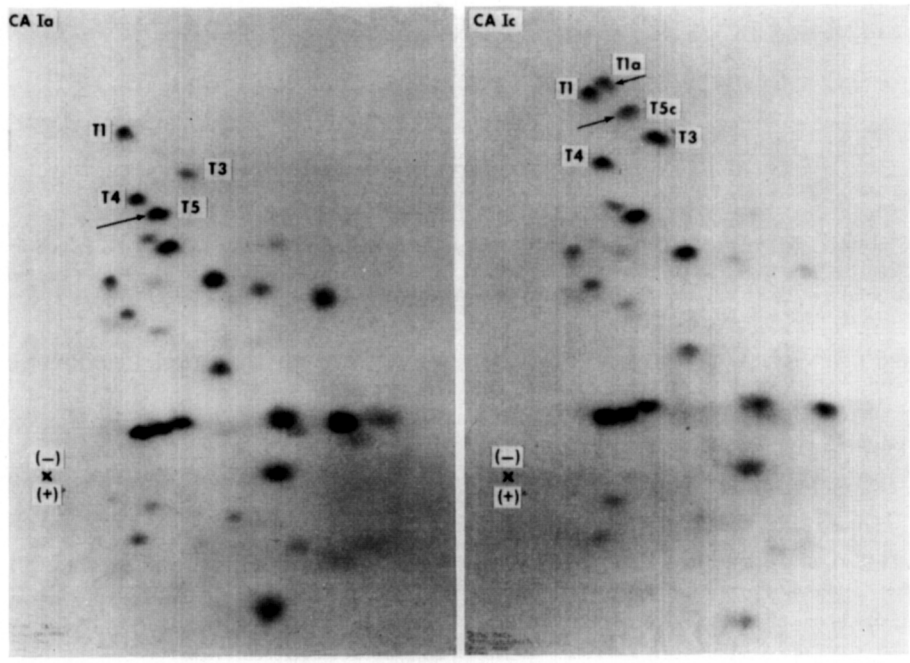

FIG. 3. Comparison of the tryptic peptide patterns of CA Ia and CA Ic. Arrows point to the peptides that are different in the two patterns.

TABLE II

COMPARISONS OF THE MOLAR RATIOS OF AMINO ACIDS IN SELECTED TRYPTIC PEPTIDES FROM CA Ia AND CA Ic

Values represent the average ratios from 3 separate isolations; residues present as 0.15 mole or less have been omitted. The assumed number of residues is given in parentheses.

Amino acid	CA Ia peptides					CA Ic peptides				
	T1	T3	T3[a]	T4	T5	T1	T1a	T3	T4	T5c
Lysine	1.05 (1)			1.04 (1)		1.00 (1)	1.04 (1)		0.90 (1)	
Arginine	0.96 (1)	1.0 (1)	1.0 (1)		1.06 (1)	0.95 (1)	0.96 (1)	1.0 (1)		2.0 (2)
Glycine	1.03 (1)			0.95 (1)	0.94 (1)	1.04 (1)			1.10 (1)	
Residues	3	1	1	2	2	3	2	1	2	2[b]
Yield	20%	5%	7%	22%	43%	17%	8%	24%	18%	30%
Ninhydrin color	Yellowish blue[c]	Blue		Yellowish blue[c]	Yellow[d]	Yellowish blue[c]	Blue	Blue	Yellowish blue[c]	Blue

[a] Not acid hydrolyzed.
[b] See text for explanation.
[c] Rapidly changing to blue.
[d] Slowly changing to blue.

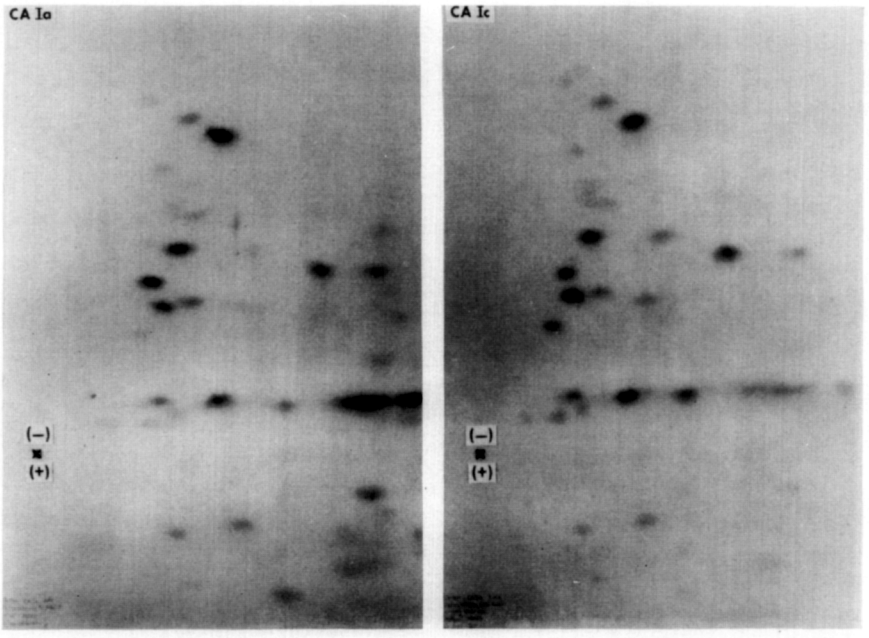

FIG. 4. The chymotryptic peptide patterns of the undigested "cores" CA Ia and CA Ic after tryptic digestion.

which favors a more positive electrical charge of this peptide.

The substitution of glycine by arginine has been previously reported for an ultraviolet-induced mutation (A-23) in the tryptophan synthetase A protein of *Escherichia coli* (16). A single base substitution of guanine by cytosine or adenine can account for this amino acid exchange on the basis of nucleotide triplets which have recently been designated to code for glycine and arginine (cf. 17).

It has been reported that the N-terminal amino acid of CA I is acetylated (18). If this is true, then peptide T5 could not be the N-terminal peptide, and it would follow that the amino acid residue adjacent to the N-terminal arginine of T5c would have to be either lysine or arginine. Thus, this mutation appears to have produced a highly basic region of 3 adjacent basic amino acid residues.

Note Added in Proof: The last 19 amino acid residues from the C-terminal end of human erythrocyte CA I have recently been sequenced by P.-O. Nyman and his co-workers at the University of Gothenburg (personal communication). The amino acid residues in positions 7, 8, and 9 from the C-terminal end were shown to be lysine, glycine and arginine, respectively. Thus, it is highly probable that the glycine which has been substituted for arginine in the CA Ic mutant enzyme is in the eighth position from the C-terminal end.

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