A WIDESPREAD SILENT POLYMORPHISM OF HUMAN CARBONIC ANHYDRASE III (31 ILE ⟷ VAL): IMPLICATIONS FOR EVOLUTIONARY GENETICS

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ABSTRACT

During amino acid sequence studies of carbonic anhydrase (CA) III, purified from a pool of human skeletal muscles, an electrophoretically undetectable (silent) variation was found at residue 31 which was either valine and/or isoleucine. To distinguish a simple allelic polymorphism from more complex models involving gene duplication, 11 separate CA III samples were purified from individuals of different age and racial backgrounds. Peptide mapping by high performance liquid chromatography and sequencing indicated that four were homozygous for 31-Val, three homozygous for 31-Ile and four were apparent heterozygotes. Since the ratio of Val/Ile at residue 31 was approximately 1.0 in the heterozygotes, the present observations are consistent with a simple allelic polymorphism model. Despite the small sample size, there are preliminary indications that the gene frequencies may differ among racial groups. The finding of this silent allelic polymorphism together with the finding of an electrophoretically detectable polymorphism of CA II permits us to test the linkage of the CA II and CA III genes which appear to have been formed by gene dupliction more than 300 million years ago. The possibility that the Val/Ile variation may represent a neutral mutation is discussed.

CARBONIC anhydrase (EC 4.2.1.1. carbonate dehydratase; CA) is a ubiquitous enzyme that catalyzes the reversible hydration of carbon dioxide (for reviews see: MAREN 1967; LINDSKOG *et al.* 1971; POCKER and SARKANEN 1978; LINDSKOG 1982). It is now known that there are at least three CA genes coding homologous isozymes (CA I, CA II and CA III) with characteristic properties (*cf.* TASHIAN *et al.* 1980; TASHIAN, HEWETT-EMMETT and GOODMAN 1983). CA I and CA II are expressed in many tissues including most mammalian erythrocytes, although CA I is absent in the red cells of ruminants, felids and some marsupials (*cf.* TASHIAN 1977; JONES and SHAW 1982). Both human red cell CA I and CA II are well characterized with respect to their amino acid sequences, three-dimensional structures, kinetic properties and inhibition by heterocyclic or aromatic sulfonamides (*cf.* TASHIAN 1977; LINDSKOG 1982; TASHIAN, HEWETT-EMMETT and GOODMAN 1983 and references therein). A number of rare and polymorphic electrophoretic variants of human red cell CA I and CA II have been documented (*cf.* TASHIAN, KENDALL and CARTER 1980). An apparently asymptomatic homozygous deficiency state for

human CA I (KENDALL and TASHIAN 1977) and, more recently, a homozygous CA II deficiency causing osteopetrosis with renal tubular acidosis and cerebral calcification (SLY *et al.* 1983) have been described.

CA III was first discovered in mammalian red skeletal muscle where it constitutes 1–2% of the soluble protein (HOLMES 1976; KOESTER, REGISTER and NOLTMANN 1977; TASHIAN 1977; CARTER *et al.* 1979). Although it is less well characterized than CA I and CA II, the amino acid sequence of bovine CA III has been determined (TASHIAN *et al.* 1980), and kinetic and inhibition studies indicate that it is a less efficient CO_2 hydratase than CA I and CA II, and it is only weakly inhibited by sulfonamides (KOESTER, PULLAN and NOLTMANN 1981; SANYAL *et al.* 1982). HEATH *et al.* (1983) have now detected, by radioimmunoassay, low levels of what appears to be CA III in human red cells. The CA III gene is also expressed at appreciable levels in rat liver (CARTER *et al.* 1981) where its synthesis is under androgenous control. Unlike CA I and CA II, no genetic electrophoretic variation of CA III has yet been detected in humans (CARTER *et al.* 1979; D. HEWETT-EMMETT, R. J. WELTY and R. E. TASHIAN, unpublished results) or mice (R. S. HOLMES, personal communication). CA II has been mapped to chromosome *8* in humans (VENTA *et al.* 1983) and to chromosome *3* in mice (EICHER *et al.* 1976). Since the CA I and CA II loci appear to be closely linked in mice (EICHER *et al.* 1976), guinea pigs (CARTER 1972) and pigtail macaques (DESIMONE, LINDE and TASHIAN 1973), the detection of genetic variation in CA III would be an important step in determining whether the CA III locus is also linked to the CA I and CA II genes. If this proves to be so, the CA multigene enzyme family would have retained close gene linkage throughout the more than 300 million years since the duplications that gave rise to these genes occurred (TASHIAN, HEWETT-EMMETT and GOODMAN 1980, 1983).

In this paper, we detail the discovery of genetic variation in human CA III during the determination of its amino acid sequence and show that the variation represents an electrophoretically silent polymorphism present in different racial groups. As far as we know, it represents the first such silent, asymptomatic human enzyme polymorphism; although, if the neutral mutation theory (*cf.* WILSON, CARLSON and WHITE 1977) has any basis, there should be large numbers of such polymorphisms (*cf. e.g.*, BOYER *et al.* 1972; KIMURA and OHTA 1971).

MATERIALS AND METHODS

Human CA III was purified from autopsied red skeletal muscle obtained by courtesy of the University of Michigan Hospital. The muscle used was psoas major which had been obtained from individuals of different age, sex and racial origins. The muscle tissues were stored at 4° if used immediately or at −20° if for future use.

Purification of CA III from red skeletal muscle: All purification procedures were carried out at 4°, and all buffers contained 1 mM β-mercaptoethanol to prevent the slow dimerization of CA III reported elsewhere (REGISTER, KOESTER and NOLTMANN 1978; CARTER *et al.* 1979). About 200 g of muscle tissue, stripped of fat, were passed twice through a meat grinder and then homogenized in a Waring blendor with 400 ml of 0.01 M Tris-SO_4, pH 8.7. The crude extract was stirred overnight and then centrifuged for 30 min at 11,000 rpm. The supernatant was dialyzed overnight

against 0.005 M Tris-SO_4, pH 8.7, and then passed twice through a sulfonamide-bound affinity (Prontosil-CM Sephadex) column (2.5 × 40 cm) which had been equilibrated with the dialysis buffer (OSBORNE and TASHIAN 1975). Low ionic strength buffer is necessary due to the weak affinity of CA III for sulfonamides. The column was washed with this buffer to remove the unbound protein. The CA III was then eluted with a linear gradient developed with 150 ml of 0.005 M Tris-SO_4, pH 8.7, and 150 ml of 0.005 M Tris-SO_4, 0.2 M KI, pH 8.7, at a flow rate of 50 ml/hr. The fractions were monitored spectrophotometrically at 280 nm, and those containing CA activity [determined by a bromthymol blue spot test (TASHIAN 1969)] were pooled and dialyzed overnight against the same buffer to remove KI, the degradation of which causes protein modification. The pool was then concentrated by vacuum dialysis to approximately 5 ml. For further purification the sample was then applied to a G-75 column (3 × 110 cm) equilibrated with 0.002 M Tris-Cl, pH 8.0. The fractions were identified as before and tested for purity by SDS-polyacrylamide gel electrophoresis (12.5% separating gel, 5% stacking gel) at 150 V, 55 mA for 3 hr (LAEMMLI and FAVRE 1973). The fractions containing pure CA III were pooled and vacuum dialyzed to approximately 2 ml. These concentrated samples were then tested for purity by the same gel system. Approximately 80 mg of CA III were isolated from each individual.

Trypsin digestion: Approximately 3.5 mg of purified human CA III was first denatured by lowering the pH to 2.0 with 1 N HCl and by raising the pH to 11.0 using 1 N NaOH. The pH was then readjusted to 8.5 with 0.2 N HCl. A 1/10 volume of 0.5 M Tris-Cl, pH 8.5, was added to buffer the digestion. Trypsin (Worthington Biochemicals Corporation, Freehold, New Jersey) was added (1/50 w/w) and the reaction was allowed to proceed for 18 hr at 37°. The digest was centrifuged, and the soluble peptides were concentrated to ~500 μl.

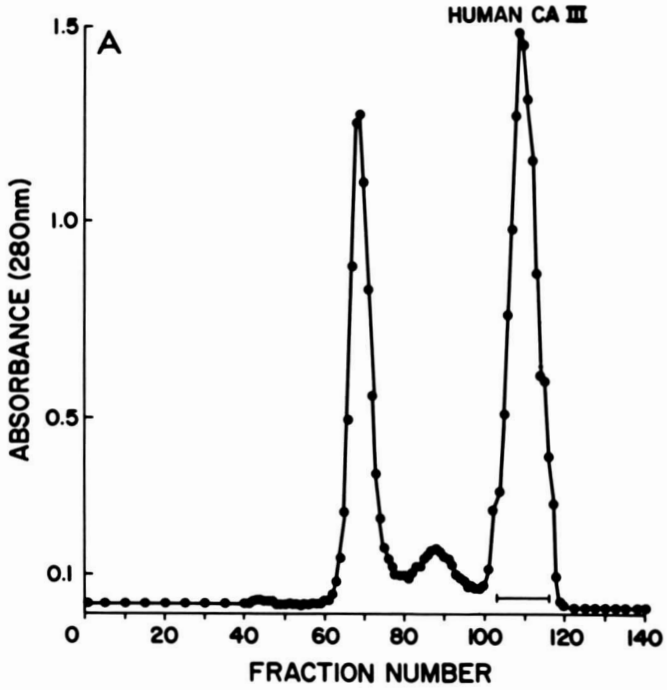
High performance liquid chromatography (HPLC) separation of the tryptic peptides: Diagnostic samples (~20 μg) were analyzed on a Waters C_{18} μBondapak reverse-phase column using a Beckman-Altex (model 332) HPLC system (HEWETT-EMMETT 1982). Buffer A was 0.1% (w/v) trifluoroacetic acid (Pierce Chemical Corporation, Rockford, Illinois) in HPLC grade water (Burdick and Jackson, Muskegon, Michigan). Buffer B was 0.05% (w/v) trifluoroacetic acid in UV grade acetonitrile (Burdick and Jackson). A linear gradient from 0 → 70% buffer B was developed in 70 min at a flow rate of 1 ml/min. Peptides were monitored at 215 nm, and a Hewlett-Packard 3390A integrator was used to estimate peak areas. To prepare peptides for sequence analysis, approximately 1 mg of the tryptic digest was loaded on the HPLC column, and a more shallow linear gradient (0 → 70% buffer B) was developed in 140 min.

Sequence analysis: The relevant peptide(s) from the tryptic digests of different CA III samples were blown dry under a stream of nitrogen, and the amino acid sequence was determined as described elsewhere (HENRIKSSON, TANIS and TASHIAN 1980; KAGEOKA *et al.* 1981).

RESULTS

CA III was purified from 11 individual human psoas major muscle samples. In addition, two pure CA III pools were analyzed. The first was a pool made up of muscles of unknown racial origin and number, and the other was from a pool of seven muscles of unknown provenance. Following elution from the sulfonamide-bound affinity column, the concentrated fractions containing CA III were applied to a G-75 Sephadex column. A typical elution profile is shown in Figure 1A. An SDS-polyacrylamide gel of the 11 purified CA III samples is shown in Figure 1B which demonstrates that the human CA III samples are approximately 95% pure.

Each sample was digested with trypsin, and the tryptic peptides were separated by reverse-phase HPLC. Examples of the various peptide patterns obtained are shown in Figure 2. The peptide pattern obtained for CA III from the pool of seven muscles is shown in Figure 2A. Unexpectedly, two peptides with similar sequence representing residues 25–36 (designated T3) were



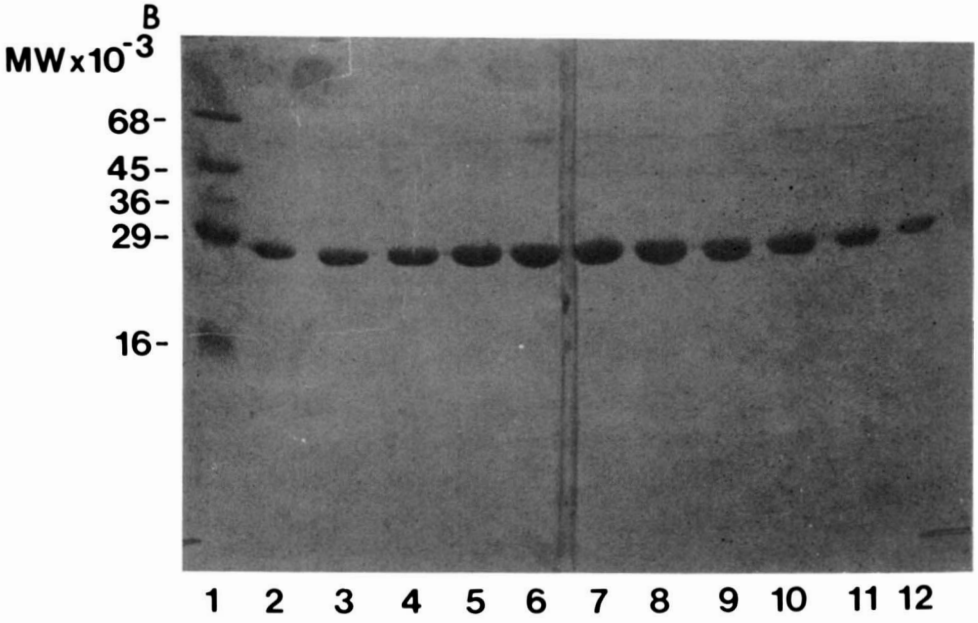


FIGURE 1.—Purification of human CA III. A, Typical gel filtration profile of a concentrated CA III sample (eluted from the Prontosil-CM Sephadex affinity column) applied to G-75 Sephadex. Early eluting peaks represent contaminating proteins that were either bound to the affinity column or to CA III. B, SDS-polyacrylamide gel electrophoresis demonstrating the purity of the 11 individual skeletal muscle CA III samples (lanes 2–12) used in the study. Lane 1 represents a series of molecular weight markers.

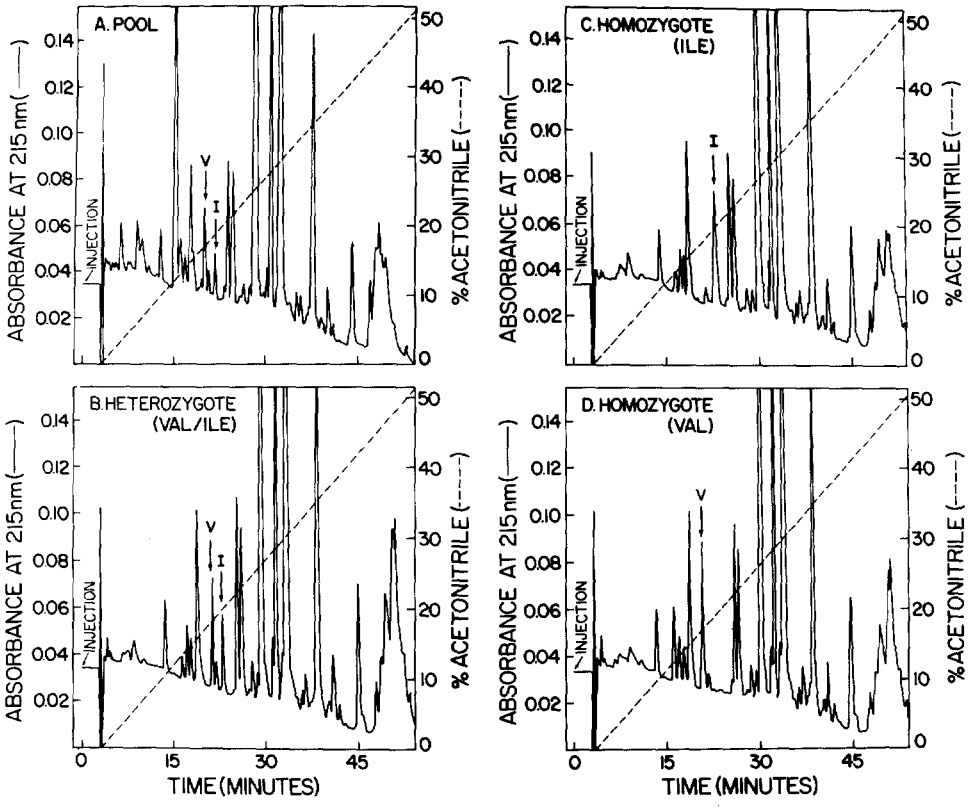


FIGURE 2.—HPLC profiles of tryptic peptides from human CA III samples. A, A pool of seven muscles showing the presence of both peptides T3 (31-Val) and T3 (31-Ile), denoted by V and I, respectively, in a ratio of approximately 2:1. B, An individual (autopsy 1 in Table 1) exhibiting both T3 (31-Val) and T3 (31-Ile) in a ratio of 1.1:1. This is a presumed heterozygote. C and D, Individuals exhibiting only T3 (31-Val) and T3 (31-Ile), respectively.

found.[1] The two peptides had the sequences:

	25				31				36
T3 (31-Val):	Gly-	Glu-	Asn-	Gln-	Ser-	Pro-	*VAL*-	Glu-	Leu-	His-	Thr-	Lys
T3 (31-Ile):	Gly-	Glu-	Asn-	Gln-	Ser-	Pro-	*ILE*-	Glu-	Leu-	His-	Thr-	Lys

The more abundant peptide, eluting earlier, had valine at residue 31, and the less abundant peptide had isoleucine at residue 31. The ratio of T3 (31-Val) to T3 (31-Ile) was approximately 2:1. In the other pool of human psoas muscles (of unknown origin and number), the reverse ratio was found, *i.e.*, T3 (31-Ile) was approximately twice the concentration of T3 (31-Val). These findings on the muscle pools were described in an earlier abstract (HEWETT-EMMETT, WELTY and TASHIAN 1982). Figure 2B–D shows the tryptic peptide profiles obtained from CA III from three different muscle samples; they represent an individual with both peptides (B), only T3 (31-Val) (C) and only T3 (31-Ile)

[1] Residues are numbered by homology with the human CA I sequence (cf. TASHIAN *et al.* 1980).

(D). Similar typing of the other eight CA III samples revealed the results shown in Table 1, which also details the age, sex and racial origins of the individuals from which the samples were obtained. The results show four individuals with only the T3 (31-Val) peptide, three with only the T3 (31-Ile) peptide and four with both peptides. From integration of the peptide peak areas, those individuals with both peptides have approximately equal quantitites of each. The HPLC elution time of the two peptides is quite repeatable; however, in each case, the peptide was collected and subsequently sequenced to confirm the identity of the amino acid at residue 31. In all cases, the sequence results agreed with the amino acid predicted from the peptide elution behavior on HPLC.

DISCUSSION

Variation at residue 31 of human skeletal muscle CA III in both blacks and whites and the finding of both homozygotes and presumed heterozygotes in both racial groups despite the small sample size indicate that it is a widespread polymorphism. The ratio of Val/Ile is close to 1.0 in the four heterozygotes examined, which is consistent with a simple allelic polymorphism. The possibility of a duplication in addition to the allelic variation cannot be entirely ruled out. However, in well-documented cases of gene duplication combined with allelic variation, peptide ratios in the "heterozygotes" are more complex (*cf.* NUTE 1974; CLEGG 1974). There are indications that the frequency of $CA3^{31\,Val}$ may be higher in blacks (P = 0.80) than whites (P = 0.33) (Table 2); but a larger sample size is needed to verify this difference. This polymorphism of human CA III shares similarity with the human haptoglobin light chain polymorphism where two alleles occur at widely differing frequencies throughout different racial and geographic groups (*cf.* GIBLETT 1969; BOWMAN and KUROSKY 1982). The Hp^2 gene ranges in frequency from 0.86 in native Sri Lankans and 0.83 in Australian Aborigines to 0.27 in Melanesians and approximately 0.30 in Liberian and Nigerian blacks. In the case of haptoglobin, the absence of a polymerizing Hp^2-like allelic product in the great apes indicates that the polymorphism results from a mutation early in the human lineage after divergence from the great apes. In the case of CA III, it is difficult to acquire comparable data relating to the antiquity of the mutation that resulted in the human polymorphism. In a comparison of CA III from human and gorilla skeletal muscle, it was originally suggested that human CA III had Ile at residue 31 and gorilla had Val (HEWETT-EMMETT and TASHIAN 1981). This work was based on single samples, and the peptides sequenced were purified by paper electrophoresis and chromatography which would not separate the two forms of peptide T3. Clearly, we were dealing with a homozygous Ile/Ile human and a homozygous Val/Val gorilla. Whether the polymorphism of CA III predated the divergence of humans and apes 5–15 million years ago (WILSON, CARLSON and WHITE 1977; GOODMAN *et al.* 1982) remains to be elucidated by studies on a series of samples from man's closest relatives (*i.e.*, gorillas and chimpanzees).

Based on the X-ray crystallographic, three-dimensional structures of human

TABLE 1

Variation at residue 31 of human skeletal muscle CA III in eleven individuals

Autopsy no.	Age	Sex	Racial origin	Phenotype	Val/Ile ratio
1	39	Male	Black	Val/Ile	1.1
2	45	Male	Black	Val/Ile	1.0
3	35	Male	White	Val/Val	
4	28	Male	Black	Val/Val	
5	18	Female	White	Val/Ile	1.2
6	50	Male	White	Val/Ile	0.9
7	23	Male	White	Ile/Ile	
8	5	Male	Black	Val/Val	
9	65	Male	Black	Val/Val	
10	43	Male	White	Ile/Ile	
11	40	Male	White	Ile/Ile	

TABLE 2

Summary of data on allelic polymorphism at residue 31

	n	Val/Val	Val/Ile	Ile/Ile	Allelic frequency of $CA3^{31\,Val}$
White	6	1	2	3	0.33
Black	5	3	2	0	0.80
Total	11	4	4	3	0.55

For explanation of CA gene nomenclature, see VENTA *et al.* (1983).

CA I and CA II (*cf.* NOTSTRAND, VAARA and KANNAN 1975), residue 31 is located in the interior of both molecules. This residue (which is Val in both CA I and CA II) is part of a small β-structure (residues 30–44) and not one of the 32 residues that have been assigned to the hydrophobic cores of these isozymes. Since the amino acid sequences of the three isozymes are almost equally divergent from one another (TASHIAN, HEWETT-EMMETT and GOODMAN 1983), and since human CA I and CA II have very similar three-dimensional structures, it is likely that residue 31 in the CA III isozyme occupies a similar position to that found in the other two isozymes. So far, in mammals, Ile and Val appear to be interchangeable at position 31 (Table 3). This may reflect the preference for an aliphatic, branched side chain adjacent to the α-carbon atom which is important for maintaining certain aspects of the secondary (or tertiary?) structures of these molecules. It is possible that because of certain steric hindrances Ile and Val, but not Leu, are acceptable at this position. In this respect, it is of interest that a comparison of amino acid changes in a series of homologous proteins shows that Val appears to change more often to Ile than to Leu (DAYHOFF, ECK and PARK 1972).

The fate of mutant alleles has provoked much controversy during the last 15 years or so; this centers around what proportion of nonharmful mutations are selectively neutral and what proportion are beneficial. Approximately 25% of all mutations do not result in an amino acid change due to the degeneracy

TABLE 3

Residue 31 in carbonic anhydrase isozymes

Species	Isozyme	Source	Residue 31
Human (*Homo sapiens*)	CA III	Skeletal muscle	Ile/Val
Gorilla (*Gorilla gorilla*)	CA III	Skeletal muscle	Val
Ox (*Bos taurus*)	CA III	Skeletal muscle	Ile
Chicken (*Gallus domesticus*)	CA III	Skeletal muscle	Ile
Human	CA I	Red cell	Val
Chimpanzee (*Pan troglodytes*)	CA I	Red cell	Val
Orangutan (*Pongo pygmaeus*)	CA I	Red cell	Val
Rhesus macaque (*Macaca mulatta*)	CA I	Red cell	Val
Ox	CA I	Rumen	Ile
Horse (*Equus caballus*)	CA I	Red cell	Val
Turtle (*Malaclemys terrapin*)	CA I	Red cell	Ile
Human	CA II	Red cell	Val
Rhesus macaque	CA II	Red cell	Val
Capuchin monkey (*Cebus sp.*)	CA II	Red cell	Val
Sheep (*Ovis aries*)	CA II	Red cell	Val
Ox	CA II	Red cell	Val
Rabbit (*Orycytolagus cuniculus*)	CA II	Red cell	Ile
Horse	CA II	Red cell	Val
Mouse (*Mus musculus*)	CA II	Spleen (cDNA)	Val

Sequences are referenced in TASHIAN *et al.* (1983) except mouse CA II (BALB/c) which is based on the cDNA sequence of CURTIS *et al.* (1983).

of the genetic code and will be detected only by DNA studies. Of the remainder, 38% will result in an electrophoretically detectable charge alteration (assuming histidine = lysine, arginine). Thus, there might be expected to be approximately twice as many electrophoretically "silent" variants as easily detectable charge-altering variants. The existence of polymorphisms presumably represents one of three possibilities: (1) heterosis (preferential maintenance of heterozygotes), (2) a transient stage during the random drift of neutral mutations and (3) a transient stage in the fixation of a mutation under selective pressure. Because of the interchangeability of Val/Ile in the evolution of the three isozymes, it seems likely that in this case we may be looking at a neutral mutation that, because of the size and complexity of the human species, is unlikely ever to be fixed. What is also interesting about human CA III is that electrophoretic variants appear to be rare compared with CA I and CA II. More is now known about the physiological roles of CA II than those of CA I or CA III, due in large measure to a recent report describing the association of CA II deficiency with disruption of bone resorption and with bicarbonate reabsorption in the kidney (SLY *et al.* 1983). However, the evolutionary conservatism of both CA I and CA III (TASHIAN *et al.* 1980) implies that they have equally important functions, although deficiency of CA I in erythrocytes of humans (KENDALL and TASHIAN 1977) and pigtail macaques, where it is polymorphic (*cf.* TASHIAN and CARTER 1976), is apparently asymptomatic. Perhaps, the rarity of electrophoretic variants in CA III is associated with its role

in skeletal muscle and suggests that it may interact with other proteins, rendering surface charge changes harmful.

The power of HPLC in detecting rare electrophoretically silent variants (SHELTON *et al.* 1982; WILSON, TARR and KELLEY 1983; STRAHLER, ROSENBLOOM and HANASH 1983) and silent amino acid changes between gene duplicates (CONGOTE 1981; STRAHLER and MEISLER 1982) has also been noted. Although intact CA III elutes as a single peak from HPLC whether from heterozygous (*i.e.*, 31 Ile/Val) or homozygous individuals, it was thought that a partial separation of CA III (31 Val) from CA III (31 Ile) was possible. To test this possibility, fractions from a heterozygous individual were collected from the first and second halves of the peak, lyophilized and digested with trypsin. The HPLC peptide patterns were identical, indicating that there was no separation of the two allelic products (D. HEWETT-EMMETT, unpublished results). This contrasts with the human $^{G}\gamma$- and $^{A}\gamma$-globin chains which also differ by only one silent amino acid change. (Gly/Ala) but can be separated by HPLC (CONGOTE 1981). This may reflect either the importance of size, since the globin γ-chains have 146 amino acids and CA III has 259 amino acids, or the fact that residue 31 is probably internal in CA III, whereas the Gly/Ala position (residue 14 of the H helix) is external in γ-globin.

The difficulty in typing CA III molecules directly, and the impracticality of doing family studies using muscle as a source material, means that other methods will have to be found in order to extend the study. The ability to purify ~1–2 mg of what appears to be CA III from 300 ml of packed red blood cells (HEATH *et al.* 1983; R. J. WELTY, unpublished results) makes family studies more feasible. Another possibility is to study the polymorphism at the DNA level by Southern blotting of suitable digested genomic DNA. This would require a CA III cDNA probe. A cDNA probe for mouse CA II (CURTIS 1983) is now available, and it has been shown to hybridize with human CA II genomic sequences (VENTA *et al.* 1983) and probably to other CA isozyme sequences. The finding of a polymorphism in the CA III gene will be important in gene mapping and linkage studies, assuming a suitable restriction enzyme can be found that distinguishes the $CA3^{31\,Val}$ and the $CA3^{31\,Ile}$ alleles at the DNA level. Since there is a polymorphism in blacks for both CA II and CA III, and also glutathione reductase (GSR) (*cf.* GIBLETT 1969), it should be possible to test the proposed gene linkage of CA II and GSR (VENTA *et al.* 1983) and the possible linkage of CA III to both if a suitable extended family can be found to provide blood for protein and DNA studies.

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