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ACP$_1^{GUA\text{-}1}$—A Low-Activity Variant of Human Erythrocyte Acid Phosphatase: Association with Increased Glutathione Reductase Activity

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SUMMARY

ACP$_1$GUA-1, a variant of human erythrocyte acid phosphatase, exists as a polymorphism (allele frequency of .132) in the Guaymi Indians of Central America. This variant has an electrophoretic mobility similar to the common B- and C-type variants, but individuals of the ACP$_1$GUA-1 phenotype have a level of enzyme activity only 27% of the activity expected for the ACP$_1$C variant. The GUA-1 variant is more thermostable than is the B variant, and the order of responsiveness to the modulation of activity by purine analogs and folate is always (B)-(A)-(GUA-1). Thus, the GUA-1 variant is a low-activity variant with C-like regulatory properties. Erythrocytes from individuals of the ACP$_1$GUA-1 phenotype have increased basal levels of glutathione reductase, and a larger fraction of the glutathione reductase protein is present as the holoenzyme, indicating increased levels of flavin adenine dinucleotide in the erythrocytes of these individuals. This is consistent with the suggestion that ACP$_1$ has a physiological function as a flavin mononucleotide phosphatase.

INTRODUCTION

Human erythrocyte acid phosphatase (ACP, E.C.3.1.3.2) occurs as a genetic polymorphism consisting of three common alleles segregating at a single autosomal locus (ACP_1). There is quantitative variation among the phenotypes of the three common alleles: ACP_1^A, ACP_1^B, and ACP_1^C, the ratio of activity being approximately 2:3:4 [1, 2]. Two other variants, ACP$_1$R and ACP$_1$TIC-1, with levels of

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activity similar to ACP_1A, occur as polymorphisms in restricted populations [3, 4]. Except for the B and C variants that are electromorphs, the variants are identified by differences in electrophoretic mobility [3–5]. Each variant also exhibits a unique thermostability profile [3, 4, 6, 7]. The activity of ACP_1 is modulated, in a phenotype-specific manner, by various purine [8, 9] and folate [10] analogs.

Although human erythrocyte ACP has been extensively employed as a genetic marker, few experiments have examined the suggested flavin mononucleotide phosphatase function of this enzyme [11]. This function could regulate the intracellular concentration of flavin coenzymes and, ultimately, flavoenzymes such as glutathione reductase. This could be the mechanism for the relationships between ACP phenotype and disease status that have been identified [12]. These include: the observation that infants of the ACP_1A and C phenotypes exhibit an increased incidence of neonatal jaundice [13–15]; similarly, that glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals with ACP_1A and C phenotypes are more susceptible to hemolytic crisis than are G6PD-deficient individuals with the ACP_1B phenotypes [16]; and that the frequency of premature birth is lowest in infants that are heterozygous for ACP_1B [17].

Here we describe the properties of a low-activity ACP_1 variant (ACP_1GUA-1) that occurs in polymorphic frequency among the Guaymi Indians of Panama [18] and Costa Rica [19] as well as the relationship between the level of ACP_1 activity and the activity of glutathione reductase (GR, E.C.1.6.4.2) assayed as an indicator of erythrocyte flavin adenine dinucleotide (FAD) concentrations [20].

MATERIALS AND METHODS

Samples

Blood samples were collected, shipped, washed, and stored as described [18, 19].

Hemolysate Preparation

Samples for use in kinetic assays were prepared by adding 6 parts lysing buffer (10 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid, 1 mM dithiothreitol, 0.05% Triton X-100, 1 mM EDTA, pH 7.0) to 1 part packed red blood cells. The samples were centrifuged at 48,000 *g* for 20 min and the supernatant stored at −76°C. Hemoglobin concentration was determined as described [21].

Packed red cells were mixed (1:1) with distilled water and subsequently extracted with toluene for preparation of hemolysates for electrophoresis [22]. The samples were centrifuged as above. The toluene-soluble fraction was discarded and the water-soluble layer stored at −76°C.

Phenotype Activity Levels

ACP was assayed by the addition of 50 μl hemolysate to 450 μl of preheated reaction mix (10 mM *p*-nitrophenyl phosphate in 50 mM [2-(N-morpholino-ethanesulfonic acid), pH 6.0]. All reactions were run at 37°C. The reactions were stopped by adding 500 μl of 5.0% trichloroacetic acid. Following centrifugation, the supernatant was diluted 1:5 with 0.5 M NaOH and the absorbance read at 415 nm. A unit of activity is 1 μmol of *p*-nitrophenol liberated/min per g hemoglobin. The molar extinction coefficient for *p*-nitrophenol was experimentally determined to be 15.5×10^3 liter · mol^{-1} · cm^{-1}. The activity of the other erythrocyte enzymes was assayed as described [21, 23, 24]. GR was assayed both in the

presence and absence of 0.1 mM added FAD. The units of GR activity are μmol product formed/g hemoglobin per hr.

Purine Modulation

The effects of selected purine analogs and folic acid on the activity of ACP were ascertained in order to group the Guaymi variant with the other "common" phenotypes: A, B, or C. To eliminate possible interference from phosphate [4, 9], and other low molecular weight molecules in the hemolysate, the samples were centrifugally desalted on Sephadex G-25 columns [25].

The assays were essentially the same as described above for phenotypic activity level. The control assay (i.e., substrate without effector) contained 50 mM acetate buffer, pH 5.5, and 8 mM *p*-nitrophenyl phosphate. The effectors were then dissolved into the assay mix at the following final concentrations: 5 mM hypoxanthine, adenine, allopurinol, or 2,6 diaminopurine, 2.5 mM uric acid, or 1.1 mM folic acid. These were all obtained from Sigma, St. Louis, Mo.

Heat Stability

Thermostability studies were conducted as described [4, 26]. Desalted samples were heated for the indicated times at 46° C, transferred to ice, and then centrifuged for 30 min at 48,000 *g*. The supernatants were assayed in acetate buffer as described above. All assays were run in duplicate.

Electrophoresis

Horizontal polyacrylamide gel electrophoresis was performed as described by Hoppe et al. [27]. 4-Methylumbelliferyl phosphate and phenolpthalein diphosphate were used in the localization of enzyme activity [22].

RESULTS

The ACP_1GUA-1 variant was first identified among the Guaymi Indians in Panama [18] and subsequently identified among members of this same tribe currently living in Costa Rica [19]. The allele frequency is .132 [19]. The electrophoretic mobility of this variant is similar to the electrophoretic mobility of the common B and C variants, but is distinguishable by the almost total absence of stainable activity in the presumptive homozygote individual (fig. 1). The electrophoretic mobility of the variant is not affected by the presence of citrate in the electrophoresis buffer (data not shown). It is not possible to distinguish between the B/B phenotype and the B/GUA-1 phenotype unambiguously on the basis of relative staining intensity following electrophoresis, although apparent differences were often detectable.

The level of enzyme activity among individuals of this electrophoretic class is presented in figure 2. A trimodal distribution is apparent. Individuals identified as ACP_1GUA-1 by the lack of significant staining intensity following electrophoresis have the lowest activity (2.36 ± 0.19 U) as was expected. This level of activity is higher than expected from the staining intensity following electrophoresis. The reason for the apparent discrepancy is unclear, although it is not caused by differences in the pH at which the assays are conducted, substrate specificity, or substrate concentration in the assay. The level of activity in the remaining individuals

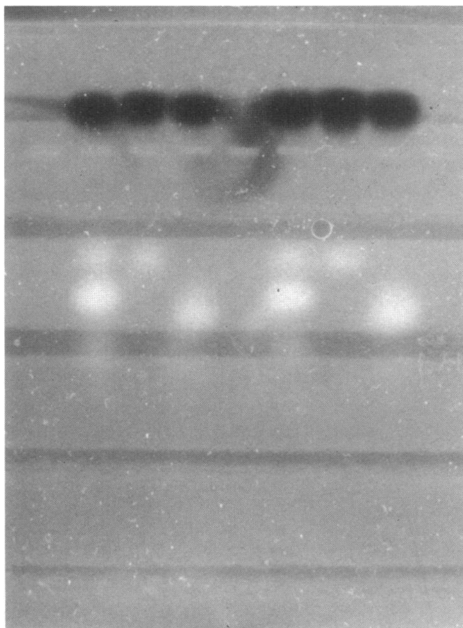

FIG. 1.—Electrophoretic pattern of erythrocyte ACP_1 variants. Electrophoretic conditions are described in MATERIALS AND METHODS. Samples were applied at *top of gel* with the cathode *at bottom*. *Phenotypes from left*: wells 1 and 4 = B, wells 2 and 5 = GUA-1, wells 3 and 6 = A.

divides into two groups with means of 4.19 ± .34 U and 6.40 ± 0.66 U. The distinction between the two groups is clear: it is assumed that individuals with ACP_1 activity between 3.4 and 5.3 U (the midpoints between the groups) are the heterozygote (B/GUA-1) individuals. These values are consistent with the existence of a low-activity, codominantly inherited allele occurring in polymorphic frequency in this population. The levels of activity for all six phenotypes existing in this population are in figure 3. The *C* allele is rarely observed in either the Guaymi [19] or among other Amerindians [28].

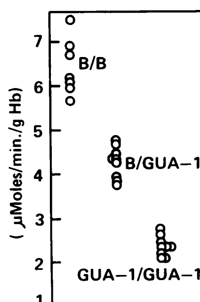

FIG. 2.—Erythrocyte ACP_1 activity in individuals with "B-like" electrophoretic phenotypes. Activity is expressed as μmol product formed/g hemoglobin/min as described in MATERIALS AND METHODS.

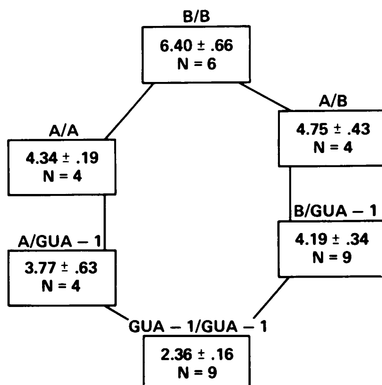

FIG. 3.—Erythrocyte ACP_1 activity in individuals of the six phenotypes existing among the Guaymi. Activity is expressed as μmol product formed/g hemoglobin/min ± SD as described in MATERIALS AND METHODS.

The level of GR activity, assayed in the absence of added FAD, was inversely related to the level of ACP_1 activity, ranging from 430 ± 135 U in erythrocytes of ACP_1B-type individuals to 680 ± 130 U in cells from ACP_1GUA-1 individuals (fig. 4). Conversely, the maximum GR activity, when the enzyme was assayed after preincubation with 0.1 mM FAD, was independent of ACP_1 activity. This latter

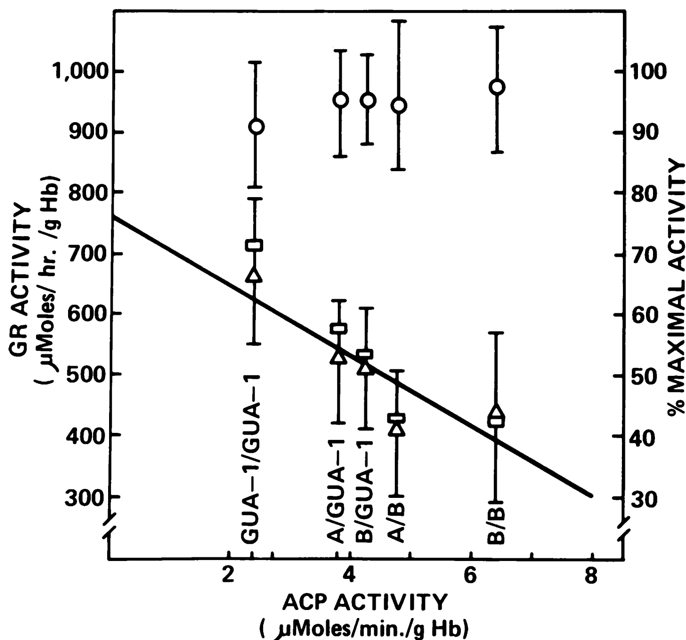

FIG. 4.—Relationship between ACP_1 phenotype and glutathione reductase activity. Δ is GR activity assayed without preincubation with FAD ± SD; O is GR activity assayed after preincubation of sample with 0.1 mM FAD ± SD; and ▭ is GR activity without FAD/GR activity in presence of added FAD × 100. All assay procedures are described in MATERIALS AND METHODS.

observation suggests that the quantity of GR protein in erythrocytes of individuals with different ACP_1 phenotypes does not differ. The activity of GR in erythrocytes assayed without preincubation with FAD and expressed as a percent of maximally stimulated GR activity is a standard measure of the erythrocyte FAD concentration [20]. As seen in figure 4, the percent active GR (▭) increases from 43% for the B phenotype to 70% in the GUA-1 individuals, indicating that low ACP_1 activity is associated with increased erythrocyte FAD levels. The activities of 12 other erythrocyte enzymes were similar to the level of activity observed in individuals collected in Ann Arbor, Michigan, [23] and were independent of the ACP_1 phenotype (activity).

The modulation of ACP activity by different purine analogs and folate is a phenotype-specific characteristic. The relative response of the A and B variants was reported [8, 9] (table 1). The activity of the B variant was doubled by the addition of hypoxanthine, while the activity of the A variant was doubled by adenine addition. Both uric acid and folic acid inhibited the A variant slightly more than they did the B variant. The B phenotype was also activated more than the A phenotype was by allopurinol and more inhibited by 2,6 diaminopurine. The activity of the GUA-1 variant was not stimulated by the addition of hypoxanthine and only slightly increased with allopurinol, but was increased threefold with the addition of adenine. The GUA-1 variant was also inhibited to a greater extent than was either the A or B variant by incubation with uric acid and folic acid, but was only 50% as responsive as the B variant to 2,6 diaminopurine. The order of responsiveness was always (B)-(A)-(GUA-1); thus, the GUA-1 variant had characteristics expected of the ACP_1C variant, which is also most responsive to modulation by adenine, uric acid, and folic acid and very unresponsive to hypoxanthine [8, 9].

The GUA-1 variant was also more thermostable than either the A or B variant (fig. 5), again a characteristic similar to that expected of the C variant [6, 7]. All three allozymes exhibited biphasic inactivation profiles when percent remaining activity was plotted on a semilog scale, but both the initial rate of activity loss and the total extent of activity loss during incubation at 46° C was least for the GUA-1 variant, while the A variant was the most thermolabile. The biphasic inactivation

TABLE 1

EFFECT OF VARIOUS COMPOUNDS WITH A PYRIMIDINYL RING STRUCTURE ON ACP_1 ACTIVITY

Compound (mM)	ACP_1B	ACP_1A	ACP_1GUA-1
Hypoxanthine	1.92 ± 0.10	1.78 ± 0.19	1.00 ± 0.19
Adenine	1.54 ± 0.18	1.93 ± 0.23	3.07 ± 0.12
Uric acid	0.79 ± 0.05	0.75 ± 0.04	0.58 ± 0.17
Allopurinol	2.11 ± 0.07	1.97 ± 0.16	1.23 ± 0.05
Folic acid	0.37 ± 0.02	0.35 ± 0.04	0.17 ± 0.01
2-6 Diaminopurine	0.18 ± 0.02	0.19 ± 0.01	0.35 ± 0.01

NOTE: Data are activity in presence of effector expressed as ratio to activity in absence of added effector ± SD. Assays were conducted as described in MATERIALS AND METHODS. No. equals 4 for each phenotype.

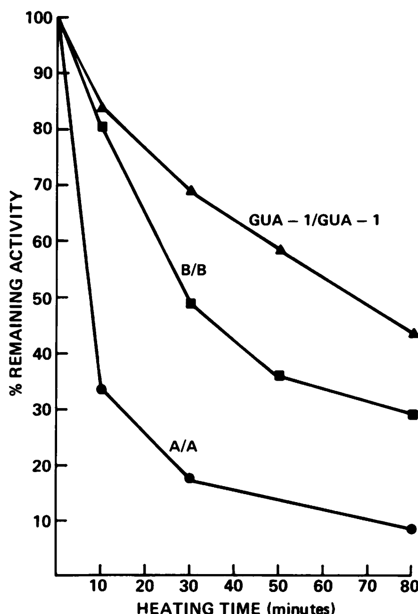

FIG. 5.—Thermoinactivation profile of several ACP_1 phenotypes. Samples (desalted hemolysates adjusted to a hemoglobin concentration of 4–5 g/100 ml) were incubated at 46°C for the times indicated. Activity was determined as described in MATERIALS AND METHODS. The % remaining activity was calculated by comparison to an aliquot maintained at 4°C. (ACP_1GUA-1 = ▲; ACP_1B = ■; ACP_1A = ●.)

profile probably reflects stability differences between the primary and secondary isozymes [7]. The $t_{1/2}$'s for the initial rate of activity loss for the A, B, and GUA-1 variants were 6, 29, and 40 min, respectively.

DISCUSSION

The ACP_1GUA-1 variant has all of the characteristics expected of the ACP_1C phenotype—electrophoretic mobility, responsiveness to purine analog modulation, and thermostability, except that the level of enzymatic activity is only some 27% of that expected in individuals of the C phenotype. This is in contrast to another ACP_1 variant that is polymorphic in another Amerindian tribe: the Ticuna [4]. The ACP_1TIC variant has a unique electrophoretic mobility and A-like levels of activity and is very thermolabile but does not exhibit a consistent modulation profile, having both A- and B-like responsiveness [4].

The function of erythrocyte ACP as a flavin mononucleotide phosphatase has been suggested by Sensabaugh [11]. This was based primarily upon the substrate specificity and kinetic properties of the enzyme. The relationship between ACP_1 activity and GR activity in this population, apparently regulated via the concentration of FAD in the erythrocyte, strongly supports this proposed function. Since GR activity is also a function of riboflavin intake [20, 29, 30], the regulatory function of ACP_1 probably can be obliterated by increasing the riboflavin intake.

The relationship between GR activity and ACP_1 phenotype (activity) in regulating erythrocyte concentrations of reduced glutathione could explain some of the ACP_1 phenotype/disease relationships previously reported [12–17]. Although the GR activity in erythrocytes from newborns is higher than in erythrocytes from an adult, a smaller proportion of the enzyme is active [24], suggesting that the erythrocytes in the newborn may be relatively more FAD-deficient than are the erythrocytes in the adult. The other enzyme in the glutathione cycle, glutathione peroxidase, is also less active in the erythrocytes from newborns [31, 32]. Additionally, the erythrocytes in newborns are more sensitive to oxidant-induced hemolysis than are erythrocytes from adults [31, 32], and the reduced glutathione is less stable in the erythrocytes of newborns than it is in adults, although the mechanism for neither the instability nor the sensitivity to oxidant-induced hemolysis is known. Thus, in red cells, which are chronically FAD-deficient, the rate of conversion of flavin mononucleotide to riboflavin may possibly be important in maintaining optimal erythrocyte function, especially in newborns.

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