The Isozymes of Glucose-phosphate Isomerase (GPI-A₂ and GPI-B₂) from the Teleost Fish Fundulus heteroclitus (L.)*

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The fish, Fundulus heteroclitus (L.), like most advanced teleosts, possesses duplicate loci for the glycolytic enzyme, glucose-phosphate isomerase (6-phosphogluconate dehydrogenase, EC 5.3.1.9). The focus for the GPI-A₂ (where GPI represents glucose-phosphate isomerase) isozyme is preferentially expressed in anaerobic tissues such as white skeletal muscle, while GPI-B₂ predominates in aerobic tissues like liver and red muscle. We questioned whether this tissue specificity would be reflected in unique structural and functional characteristics of the respective isozymes. Consequently, an analysis of the two isozymes was undertaken. The enzymes were purified by a combination of ion-exchange chromatography and isoelectric focusing. Each isozyme was characterized as native and subunit molecular weight, isoelectric pH, and susceptibility to thermal denaturation. Both were dimeric enzymes, with native molecular masses of 110 kDa. The isoelectric pH values for GPI-A₂ and GPI-B₂ were 7.9 and 6.4, respectively. Differences were apparent in thermal stability, i.e. GPI-A₂ was more stable than GPI-B₂.

Kinetic properties were investigated as a function of both pH and temperature. The $K_v$ values for fructose 6-phosphate (Fru-6-P) differed between the isozymes at low pH, but no significant differences were observed at higher pH. The inhibition constant ($K_i$) for 6-phosphogluconate (6-P-gluconate) was pH dependent. GPI-A₂ was slightly more sensitive to 6-P-gluconate inhibition than GPI-B₂ between pH 7.0 and 8.5. The $K_v$ for Fru-6-P was temperature dependent for the GPI-B₂ isozyme, but relatively temperature independent for GPI-A₂ between 10 and 35 °C. The $K_i$ for 6-P-gluconate was temperature dependent for both isozymes. The $K_v$ values for GPI-A₂ were consistently lower than those for GPI-B₂. Energetic activation differed between the two isozymes by 4.4 kcal with GPI-A₂ having the lower value. While $\Delta G\#$ values were identical for the isozymes, their $\Delta H\#$ and $\Delta S\#$ values differed significantly. The structural and kinetic differences that exist between the glucose-phosphate isomerase iso-

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Since the recognition of the biological significance of isozymes (1), they have been widely used as probes for biochemical, developmental, physiological, and evolutionary studies (2). The role of gene duplication as the origin of isozymic systems has been the subject of much discussion (3–9). Major advances in vertebrate evolution are thought to have occurred largely through gene duplications which increase the genome size. The evolution of initially redundant DNA into new genetic information is thought to have facilitated the radiation of the vertebrate line (5). Support for this theory comes from several independent observations: the nonlinkage of many homologous isozyme loci (10–12), karyotype and linkage group conservation of fishes (13–15), and the concurrent appearance of multiple isozyme loci for several different enzymes in fishes (9).

In general, advanced teleosts have more duplicated loci than do land vertebrates (9). For example, surveys of diverse species (6, 16) have shown that most possess a single locus for glucose-phosphate isomerase, while advanced bony fishes have multiple glucose-phosphate isomerase loci (6, 11, 16). The only other group to exhibit two or more glucose-phosphate isomerase loci are the dicotyledonous plants (17–19).

Glucose-phosphate isomerase isozymes of fish are thought to have arisen by a gene duplication event that took place nearly 180 million years ago during the Jurassic period (6). Other studies (9) have estimated the time of gene duplication to be even earlier. Gene duplication is thought to be a prerequisite for divergence and specialization of gene function (3–9). Current theory states that following gene duplication, one gene product generally retains the original function while the second gene undergoes additional mutations allowing a new or modified protein function (4, 5, 8). Alternatively, parallel modifications of isozymic functions can be accommodated if they are not too drastic.

The biologically significant divergence of glucose-phosphate isomerase isozymes is implied by strikingly different tissue distribution. In most higher teleosts, one $G_{pi}$ locus is primarily expressed in white skeletal muscle, while the other predominates in liver and most other tissues and internal organs (20–22). This pattern of tissue specificity is observed not only for the $G_{pi}$ locus but for many other loci as well (9, 23). Isozyme tissue specificity suggests that these enzymes have adapted through evolutionary processes toward the specific metabolic needs of the tissues in which they are expressed.

The existence of two $G_{pi}$ loci in the fish Fundulus hetero-
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lipid predominates in anaerobic white skeletal muscle, while GPI-clitus was first reported by Avise and Kitto.

In an effort to test this hypothesis, the enzymes were purified, characterized, and their steady-state kinetics were examined. Since environmental temperature has a direct effect on the internal temperature and pH of eutechters (24–29, 60–62) and these parameters strongly influence the catalytic behavior of enzymes (30, 31), the effects of these variables on the glucose-phosphate isomerase isozymes were studied. The data indicate that the two isozymes have diverged and suggest they have become functionally adapted to the tissues in which they are expressed.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

**Purification**—Summaries of the purification schemes for the liver and muscle isozymes are given in the Miniprint (Tables I and II, respectively). Fig. 1 (in Miniprint Section) shows a representative profile of the isoelectric focusing column used in the purification of GPI-A.

Similar results were obtained for the liver isozyme. The isolation procedure gave yields between 7 and 12%. Methods developed for isolation of glucose-phosphate isomerase from mammalian systems (32–34) were not effective in the purification of Fundulus glucose-phosphate isomerase. The use of phosphocellulose, CM-cellulose, or 8-(6-aminohexyl)amino-ATP-Sepharose columns in various combinations with substrate (Glc-6-P, Fru-6-P, or 6-P-gluconate) and/or salt elutions occasionally showed promise, but in general were not reproducibly effective in the purification of Fundulus glucose-phosphate isomerase.

**Criteria for Purity**—The following criteria were sought to establish the purity of each preparation: 1) homogeneity on the basis of charge as assessed by polyacrylamide gel electrophoresis and isoelectric focusing; 2) homogeneity by molecular size and mass as assessed by electrophoretic and gel filtration behavior; 3) homogeneity on the basis of constant specific activity for each enzyme across the peak during final gel filtration characterization; and 4) constancy of specific activity upon the employment of further fractionation procedures.

Purity of 95–98% was obtained for most GPI-A and GPI-B preparations; however, occasionally 92–95% was obtained for the less stable GPI-B enzyme.

**Molecular Mass**—Some physical characteristics determined for the glucose-phosphate isomerase isozymes are summarized in the Miniprint (Table III). Molecular masses for the muscle and liver isozymes determined by gel filtration were 110 ± 5.0 kDa. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yielded a single band between 54 and 55 kDa. These data suggest that the isozymes are dimeric proteins. Molecular masses of native glucose-phosphate isomerase in other species range from 107 to 132 kDa, while subunit molecular masses of 54–67 kDa have been reported (17–20, 32, 33, 35–40). Thus the glucose-phosphate isomerase isozymes from *F. heteroclitus* have molecular masses typical of those isolated from other organisms.

**Isoelectric Point Determinations**—The isoelectric pH of each isozyme (see the Miniprint, Table III) was obtained from the isoelectric focusing column used as the last step in purification. The average of several pH determinations gave the following results for GPI-A: 7.0 ± 0.1 and GPI-B, 6.6 ± 0.1. Isozymes from catfish show similar pH values of 7.0 for muscle and 6.2 for the liver isozymes (20). Glucose-phosphate isomerase values from other sources vary widely in their isoelectric pH values. In general, mammalian glucose-phosphate isomerase values are more basic than those of teleosts (20, 33, 34, 41).

**Effect of pH on Stability of Glucose-phosphate Isomerase**—The effect of pH on stability of glucose-phosphate isomerase muscle and liver isozymes is shown in Fig. 2 (Miniprint). Both isozymes are unstable at low pH (<pH 6.5). Stability was maintained during long-term storage by concentrating the sample in high pH buffer (pH 8.0), adding glycerol (40% v/v), and storing at −20°C. Because of the marked instability at low pH, catalytic studies were restricted to pH 7.0 and above.

**Heat Denaturation Studies**—Results of the heat denaturation experiments are shown in Figs. 3 and 4. The T90 values, or temperatures at which half the original enzyme activity remains, are 52.1 ± 0.1 and 42.3 ± 0.5°C for GPI-A and GPI-B, respectively. These values were not significantly altered in the presence of fructose 6-phosphate or phosphate buffer. Thus, neither the inclusion of substrate nor incubation in higher ionic strength buffer appeared to affect the thermal stability. (Details are given under "Experimental Procedures" in the Miniprint.) The differences in thermal stability were even more apparent when the enzymes were incubated at 42°C and activity monitored as a function of time (Fig. 4). The kinetics of denaturation are consistent with a simple first-order process. The muscle isozyme (GPI-A) retained virtually 100% activity after 60 min of incubation at 42°C, while GPI-B lost 50% of its activity after only 15 min.

The existence of a more stable muscle isozyme in *Fundulus* is consistent with results reported for glucose-phosphate isomerase isozymes in several other fish species (6, 16, 20, 38). Only in catfish was the muscle isozyme more labile than the liver form (20). In many of these studies, the isozyme (or allelic isozyme) with the lowest isoelectric pH was the least stable (6, 16, 20, 38). A report by Dice and Goldberg (42) of in vivo degradation rates of acidic and basic proteins in rat tissues has shown that the isoelectric points of over 22 soluble proteins from *Escherichia coli* appears to have an active tetrameric form of glucose-phosphate isomerase, Mr 220,000, in addition to the dimeric form (40).
Fig. 3. Thermal denaturation of F. heteroclitus glucose-phosphate isomerase isozymes. Aliquots of purified enzyme were incubated for 10 min at various temperatures as described under "Experimental Procedures" in the miniprint. The percent of the residual activity is plotted against incubation temperature. O, GPI-A2; △, GPI-B2.

The different isozymes of Fundulus glucose-phosphate isomerase are inhibited to the same extent by high levels of Fru-6-P. The degree of inhibition decreases with increasing temperature at approximately the same rate as the $K_a$ for Fru-6-P. Thus, inhibitory levels of Fru-6-P remain relatively constant at 2–2.5 times the $K_a$. These findings are comparable to those reported for glucose-phosphate isomerase isozymes from another teleost, Astyanax mexicanus (6). The authors observed substrate inhibition for both muscle and liver isozymes at substrate levels above 200 $\mu$M Fru-6-P or roughly 2.5 times the $K_a$ for Fru-6-P.

The Effect of $pH$ on Kinetic Parameters—The dependence of $K_a$ on $pH$ varied between the isozymes at low $pH$, but no significant differences were observed at higher $pH$ values (Table IV). The sensitivity of $V_{max}$ to $pH$ is also illustrated in Table IV. Both enzymes show qualitatively similar $pH$ optima with a maximum at $pH$ 8.5 for the muscle isozyme and a slightly broader optimum range for the liver isozyme ($pH$ 8.5–9.0). Significant quantitative differences exist between the isozymes at all $pH$ values which may reflect apparent specific activity differences of the purified enzymes.

proteins was significantly correlated with degradative rates. The more stable GPI-A2 isozyme has a $pI$ more than 1 $pI$ unit higher than that of GPI-B2. If we were to assume that the rate of synthesis for both isozymes in vivo was the same, then an increased rate of degradation for the liver isozyme would result in a lower in vivo concentration of active enzyme. Support for this hypothesis comes from studies of the Singh variant of glucose-phosphate isomerase isolated from human and blood cells (34, 43). Glucose-phosphate isomerase activity in red cell lysates of these variants was significantly higher than that of normal controls. The greater in vivo activity of the variant glucose-phosphate isomerase was correlated with a greater stability to heat denaturation in vitro.

Further indication of the possible physiological importance of the heat stability differences is the wide thermal tolerance of F. heteroclitus. Studies of several Fundulus species have shown that F. heteroclitus is the most thermally tolerant (44, 45).

While the $T_{50}$ values of most enzymes are usually much higher than the upper lethal temperature of the species (30), this is not true of the glucose-phosphate isomerase system in Fundulus. The $T_{50}$ values for the isozymes range from to 42.3 to 52.1 °C. Summer temperatures in shallow marsh ponds may often approach this temperature range.

Substrate Inhibition—The results of the substrate inhibition studies are shown in Fig. 5. Substrate inhibition was not observed until the concentration of Fru-6-P exceeded twice that of the $K_a$ (40 $\mu$M at $pH$ 8, 25 °C). No difference in the extent of inhibition was observed between the isozymes. All other catalytic studies were done using noninhibitory concentrations of Fru-6-P.

Cleland (45) has suggested that substrate inhibition in a one-substrate system (like glucose-phosphate isomerase) occurs by either the dead-end combination of substrate with an inactive enzyme or the simultaneous binding of two substrate molecules in a single active site. The extent of the inhibition may be modified by different experimental parameters such as temperature, ionic strength, or $pH$.
The variances of individual experiments range from 0.3 \times 10^{-4} to 3.0 \times 10^{-4}, as calculated by the Creland program.

| pH  | Muscle isozyme, GPI-A<sub>2</sub> | Liver isozyme, GPI-B<sub>2</sub> |
|-----|----------------------------------|----------------------------------|
| 7.0 | 14.31 ± 3.43                     | 20.40 ± 2.38                     |
| 7.5 | 25.66 ± 2.05                     | 21.0 ± 2.94                      |
| 8.0 | 27.15 ± 2.38                     | 35.36 ± 4.80                     |
| 8.5 | 31.00 ± 3.35                     | 54.51 ± 7.78                     |
| 9.0 | 28.38 ± 4.21                     | 33.00 ± 3.53                     |

Cleland’s (78) least squares method as described in the Miniprint. However, a representative Dixon plot is also included in the Miniprint (Fig. 6) to graphically illustrate the data. The pH dependence of log \( K_a \) is linear for both isozymes. There are significant differences in sensitivity to inhibition at lower pH values (pH 7.0-8.6) with the muscle isozyme (GPI-A<sub>2</sub>) having a lower \( K_a \) for 6-P-glucuronate than the liver isozyme (GPI-B<sub>2</sub>). However, at pH values greater than 8.5, there are no significant differences.

The Effect of Temperature on Kinetic Parameters—The temperature dependence of \( K_a \) for Fru-6-P is shown in Table V. The \( K_a \) for GPI-A<sub>2</sub> appears to be relatively temperature independent between 10 and 35 °C. For the liver isozyme (GPI-B<sub>2</sub>), \( K_a \) shows a positive correlation with temperature.

The inhibition constant for the competitive inhibitor 6-P-glucuronate also showed strong temperature dependence (Table V). Significant differences were apparent between the muscle and liver isozymes at all temperatures. The muscle isozyme had a lower inhibition constant, i.e. a higher affinity for 6-P-glucuronate, than did the liver isozyme (Table V).

The Arrenius plots for both enzymes were linear (Fig. 7, Miniprint). Energies of activation (\( E_a \)) calculated from the graphs are summarized in Table VI. The muscle isozyme (GPI-A<sub>2</sub>) had a lower \( E_a \) than the liver isozyme (GPI-B<sub>2</sub>) by 4.4 kcal. The enthalpy (\( \Delta H^\circ \)) and entropy (\( \Delta S^\circ \)) of the transition lower state were calculated by least squares analysis of these data. The enthalpy value for the muscle isozyme is 5 kcal lower than the liver isozyme. The muscle isozyme has a relatively large negative entropy value, \(-14.6 \pm 0.6\) e.u. Although the contributions from the entropy and enthalpy terms varied between the isozymes, the \( \Delta G^\circ \) values were not significantly different.

Specific Activities—The apparent \( V_{max} \) differences between the glucose-phosphate isozymes illustrated in Tables IV and V could be the result of unique catalytic rate constants (\( k_{cat} \)) or they could arise due to different amounts of active enzyme ([\( E_a \)]) because \( V_{max} = k_{cat} [E_a] \). Even though these enzymes are of equal purity, one cannot calculate their respective \( k_{cat} \) values directly by dividing \( V_{max} \) by enzyme concentration ([\( E_a \)]) because no assurance can be given that all the protein is active enzyme. This is particularly important since these isozymes have different thermal stabilities. However, the different slopes of the respective Arrenius plots (i.e. different \( E_a \) and \( \Delta H^\circ \) values, Fig. 7) strongly suggest that there are real differences in the respective \( k_{cat} \) values for the GPI-A<sub>2</sub> and GPI-B<sub>2</sub>. If there were only differences in [\( E_a \)], the Arrenius plots would be identical or parallel and the \( E_a \) and \( \Delta H^\circ \) values would not differ significantly. Since Table VI demonstrates significant differences in \( E_a \) and \( \Delta H^\circ \), the most logical explanation would be that the isozymes have different catalytic efficiencies over the temperature range examined.

On the other hand, one could argue that the glucose-phosphate isomerase exists in the following equilibrium,

\[
E_a \leftrightarrow E_{inactive} \leftrightarrow E_{denatured}
\]

where \( E_a \) = active enzyme and \( E_{total} = E_a + E_{inactive} + E_{denatured} \). If the various rate constants for the GPI-A<sub>2</sub> and GPI-B<sub>2</sub> enzymes were differentially affected by temperature, differences in \( E_a \) and \( \Delta H^\circ \) could be explained by differential changes in \( E_a \) with temperature. While this hypothesis is logical, our heat denaturation data are not consistent with that notion. First, the slopes of the Arrenius plots converge at high temperatures, and the most heat-labile enzyme (GPI-B<sub>2</sub>) has the greatest \( \Delta H^\circ \). If there were less active enzyme (\( E_a \)) for GPI-B<sub>2</sub> than GPI-A<sub>2</sub> as the temperature increased, the slope of the GPI-B<sub>2</sub> Arrenius plot should be less than that of GPI-A<sub>2</sub>, and one might even expect it to become nonlinear at higher temperatures. This was not the case. Secondly, a denaturation scheme like that proposed above would most likely result in second or higher order heat denaturation kinetics. However, the kinetics were always first order. Therefore, the most logical and simple explanation is that there are real differences in the catalytic efficiencies of the GPI-A<sub>2</sub> and GPI-B<sub>2</sub> isozymes. However, further studies such as active site titration are necessary to verify this point and determine the critical \( k_{cat} \) values.
The specific activities of the purified Fundulus glucose-phosphate isomerase isoforms are comparable to those reported for catfish and conger eel (20). In general, glucose-phosphate isomerases isolated from teleosts at first appear to have lower specific activities than the homologous mammalian proteins (32–34, 41, 46). However, such comparisons of specific activities between homologous glucose-phosphate isomerases are difficult to evaluate due to wide variation in assay conditions (i.e. pH and temperature). Specific activities of mammalian glucose-phosphate isomerases are usually reported between 30 and 37 °C, while data for most ectotherms are reported at lower temperatures (i.e. 20–25 °C).

The literature values for the specific activities of glucose-phosphate isomerase from mammalian sources range from 750–1160 e.u./mg of protein. Specific activities from ectotherms range between 340 and 690 e.u./mg of protein. While the data presented in this paper (Table I, Miniprint) are consistent with lower activities of ectothermic enzymes, the interspecific differences between specific activities are less pronounced when comparable temperature and pH values are employed (see Table V).

The apparent specific activity of the GPI-A2 isozymes is nearly double that of the GPI-B2 isozyme. This may be due to differential stability during the assay and/or the isolation procedure. Alternatively, it may reflect the relatively higher degree of glycolytic activity in the white muscle. Studies reviewed by Newstolme and Start (47) on the maximum activities of glycolytic enzymes in different tissues reported more than twice the glucose-phosphate isomerase activity in anaerobic white muscle than aerobic tissues such as heart and brain.

Mechanism of Glucose-phosphate Isomerase-catalyzed Isomerization—Many studies have investigated the mechanism of isomerase reactions (48–58). Most isomerases show an extraordinary uniformity in many aspects of their reaction mechanisms. Glucose-phosphate isomerase, in addition to its role in aldose-ketose interconversion, is unique in that it also catalyzes the mutarotation of Glc-6-P, Fru-6-P, and in yeast, Man-6-P (mannose-6-phosphate) (57). Both the α and β anomers are used as substrates, although the β anomer is more reactive. However, there is no agreement on whether muscle and yeast glucose-phosphate isomerase have identical mechanisms of amionization nor has it been established unequivocally that all glucose-phosphate isomerases are capable of Man-6-P anomerization.

The isomerase reaction is thought to involve the transfer of a proton between carbon 1 and carbon 2. This is accompanied by the formation of a cis-enediol intermediate. Deuterium exchange experiments using glucose-phosphate isomerase isolated from human red blood cells, rabbit muscle, and yeast (58) have established that the base involved in proton transfer contains no exchangeable hydrogen atoms. Rose (55) suggests that a glutamic carboxyl group is the proton donor.

Results of the kinetic studies of Dyson and Noltmann (48) using rabbit muscle glucose-phosphate isomerase have shown that the base involved has a pKᵢ value of 6.8 which is consistent with those found in other protein molecules for imidazole groups. They suggest the participation of a histidine residue in the interconversion of Glc-6-P and Fru-6-P. Close agreement between this pK value for the free enzyme in both reaction directions implies that only the single base is involved. Recent studies by Noltmann’s laboratory (51, 52) have focused on the use of covalent active-site reagents in an attempt to elucidate the amino acids at the active site and suggest that an arginine residue may also be involved. An alternative mechanism based on crystallographic work on pig skeletal muscle (56) suggests a concerted acid-base catalysis of ring opening, with proton transfer following the binding interaction.

Our results on F. heteroclitus glucose-phosphate isomerase could be interpreted to agree with those of Dyson and Noltmann (48). A pK value for the liver isozyme, GPI-B₂, is in the general area of pH 7.5. Although this value is higher than that reported for rabbit muscle (pK = 6.94 at 30 °C), it is still within the range of pK values for an imidazole residue (59).

The pK for GPI-A₂ must be lower than that of GPI-B₂. However, due to the instability of the enzyme at low pH, the pK for GPI-A₂ could not be established (see Table IV). The differences in pK between the isozymes may be viewed in terms of “al pha stat regulation,” the conservation of the fractional dissociation state of histidine imidazole groups (αimid) where (i.e. muscle). This would tend to stabilize its αimid and optimize its buffering capacity. The relatively high pK of liver glucose-phosphate isomerase and the low pK values of Fundulus and rabbit muscle glucose-phosphate isomerase are consistent with this hypothesis.

Glucose-phosphate Isomerase Isozymes in F. heteroclitus: Physiological Implications—The trend of evolutionary development of isomerase systems in teleosts has been described by several investigators (4, 6, 9). After gene duplication, there is a period of generalized expression of both loci followed by functional specialization and then restricted expression of each locus to one or a few tissues. The tissue-specific glucose-phosphate isomerase isoforms of most teleosts are thought to have arisen through such a gene duplication event some time during the Jurassic period (6). Both earlier studies (6, 20, 21, 63) and the results of the present paper indicate that the isozymes have undergone significant divergence in (a) tissue specificity, where GPI-A₂ predominates in liver and other internal organs; (b) thermal stability, where GPI-A₂ is most tolerant of thermal denaturation; and (c) in their catalytic and thermodynamic parameters.

The present investigation of the kinetic parameters of GPI-B₂ has shown that its Kₘ for Fru-6-P has a strong positive correlation with temperature. Somero and Hochachka (64) have called this effect “positive thermal modulation.” Enzymes that are primarily dependent on ionic or electrostatic interactions in binding substrate generally show a positive correlation between temperature and Kₘ. The trend is for enhanced binding at lower temperatures which helps to “compensate” for the overall reduction in thermal energy. GPI-B₂ exhibits a Kₘ for Fru-6-P that is relatively independent of temperature over the range of 10–35 °C, but sharply increases above 35 °C (Table V). Very rapid changes in Kₘ have been found to occur at temperatures near a species’ lethal limits (64). Interestingly, the lethal temperature for adult F. heteroclitus is approximately 36 °C under isosmotic conditions (66).

Our studies have revealed some significant differences between GPI-A₂ and GPI-B₂ in several other kinetic parameters. Vmax and Vmax/Kₘ are significantly higher for the muscle
isoenzyme (GPI-A₂) while its inhibition constant for 6-P-gluconate is significantly lower.

White vertebrate skeletal muscle obtains its energy primarily from anaerobic glycolysis, especially during times of stress (47). One would expect a relatively active glycolytic system in white muscle tissue. Studies reviewed by Newsholme and Start (47) on the maximum activities of glycolytic enzymes in different tissues have found relatively high activity in white muscle, more than twice the glycolytic activity found in heart (red muscle) or brain tissue. The 2-fold higher $V_{\text{max}}$ and $V_{\text{max}}/K_m$ for GPI-A₂ than for GPI-B₂ may reflect a higher degree of glycolytic activity in white muscle tissue than in red muscle, liver, and other internal organs.

The low $K_m$ for 6-P-gluconate reported for GPI-A₂ may also reflect an adaptation specific to white muscle tissue. Pentose shunt activity is virtually absent in white muscle tissue (68), and thus only very low levels of 6-P-gluconate are expected to be present. Our studies on substrate levels in fish acclimated to high oxygen pressures (59) and Charles Montague and Chris Wertz. Acknowledgments—We thank Steve Palumbi, Michelle Queen, and Jonathan Swift for their invaluable help. We thank Shinnam Chua and Charles Montague for their assistance with the computer analysis. We thank Dr. Drew Browa, Dr. Robert Cashon, Dr. Leonard D'Michele, Dr. Mitchell Hobiah, Ira Ropson, and others for their comments and suggestions. We appreciate the artistic assistance of Dianne Powers and the secretarial assistance of Diane Warr and Chris Werte.

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**EXPERIMENTAL PROCEDURES**

**Chemicals** - The following were purchased from Sigma Chemical Co. (St. Louis, MO): glucose-phosphate (Lot 6700), Grade I, phosphoglucomutase phosphatase (Dh20), Grade I, phosphoglucomutase (Lot 6700), Grade I, thioglycol acid (Dh20), Grade I, thiourea (Lot 6700), Grade I, thiourea (Lot 6700), and thiourea (Lot 6700). All reagents were stored at -20°C and used within 24 hours of use.

**Supplementary Material** - The isozymes of glucose-phosphate isomerase (GPI-A2 and GPI-B2) from the teleost fish Fundulus heteroclitus (L.) were isolated and characterized. The isozymes were purified by a combination of chromatography and electrophoresis. GPI was purified by DE-52 chromatography, followed by QAE-Sephadex chromatography, and finally by DEAE-Cellulose chromatography. The purified GPI was then assayed for activity.

**EXPERIMENTAL PROCEDURES**

**Glucose-phosphate isomerase** - The purified enzyme was stored at -20°C in 0.05 M Tris-HCl, pH 8.0, and 0.05 M NaCl, which had been equilibrated in the same buffer. GPI was eluted by a linear salt gradient (0.0 to 0.1 M NaCl) from a DE-52 column (4.5 x 10 cm) that had been equilibrated in the same buffer. The enzyme activity was monitored by measuring the change in absorbance at 412 nm using a Beckman DU-2 spectrophotometer.

**QAE-Sephadex chromatography** - The eluate from the DE-52 column was concentrated by ultrafiltration and dialyzed against 0.05 M Tris-HCl, pH 7.5, and 0.05 M NaCl. The dialyzed sample was applied to a QAE-Sephadex column (2.6 x 54 cm) that had been equilibrated in the same buffer. GPI activity was eluted with a linear salt gradient (0.0 to 0.1 M NaCl) from the column and monitored at 280 nm using a Beckman DU-2 spectrophotometer.

**DEAE-Cellulose chromatography** - The dialyzed sample was applied to a DEAE-Cellulose column (2.6 x 28 cm) that had been equilibrated in the same buffer. GPI activity was eluted with a linear salt gradient (0.0 to 0.1 M NaCl) from the column and monitored at 280 nm using a Beckman DU-2 spectrophotometer.

**Electrophoresis** - The purified GPI was subjected to electrophoresis on a 5% acrylamide gel to further characterize the enzyme. The gel was run at 100 V for 2 hours and stained with Coomassie blue. The electrophoresis pattern was then visualized and photographed.

**References** - The purified GPI was stored at -20°C in 0.05 M Tris-HCl, pH 8.0, and 0.05 M NaCl, which had been equilibrated in the same buffer. GPI was eluted by a linear salt gradient (0.0 to 0.1 M NaCl) from a DE-52 column (4.5 x 10 cm) that had been equilibrated in the same buffer. The enzyme activity was monitored by measuring the change in absorbance at 412 nm using a Beckman DU-2 spectrophotometer.

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Glucose-phosphate Isomerase Isozymes

Table I: Summary of Isolation of Glucose-phosphate Isomerase from "Fusobacterium heteroclitum" Liver (GPI-b)

| Fraction | Total activity (enzyme units) | Total protein (mg) | Specific activity (enzyme unit/ mg protein) | Purification (overall) | Recovery (overall %) |
|----------|-------------------------------|-------------------|-------------------------------------------|----------------------|---------------------|
| I Crude homogenate | 3050 | 1799 | 1.71 | (100) |
| II Ammonium sulfate | 2052 | 1730 | 1.19 | 0.7 | 67 |
| III DEAE-Cellulose chromatography | 2164 | 80.3 | 26.9 | 16 | 71 |
| IV DEAE-Biogel chromatography | 2158 | 11.2 | 152.0 | 89 | 71 |
| V Ion Exchange Focusing | 209 | 0.8 | 260 | 152 | 7 |

Table II: Isolation of Glucose-phosphate Isomerase from "Fusobacterium heteroclitum" White Skeletal Muscle (GPI-a)

| Fraction | Total activity (enzyme units) | Total protein (mg) | Specific activity (enzyme unit/ mg protein) | Purification (overall) | Recovery (overall %) |
|----------|-------------------------------|-------------------|-------------------------------------------|----------------------|---------------------|
| I Crude homogenate | 2772 | 5705 | 0.49 | (100) |
| II Ammonium sulfate precipitate | 3260 | 4950 | 0.63 | 1 | 15 |
| III QAE-Sephadex chromatography | 925 | 95.5 | 9.29 | 19 | 33 |
| IV DEAE-Biogel chromatography | 746 | 29.5 | 29.3 | 50 | 26 |
| V Ion Exchange Focusing | 530 | 1.78 | 298 | 61 | 19 |
| VI Ion Exchange Focusing | 300 | 0.56 | 571 | 76 | 8 |

Table III: Summary of the Physical Parameters Determined for GPI-a and GPI-b

| Parameter | GPI-a | GPI-b |
|-----------|-------|-------|
| Molecular mass (KDa) | 110 ± 5.0 | 110 ± 5.0 |
| Subunit mass (KDa) | 56.6 ± 0.30 | 53.8 ± 0.70 |
| Isoelectric pH (pI) | 7.9 ± 0.1 | 6.6 ± 0.1 |
| Tgi for thermal denaturation | 52.1 ± 0.5°C | 62.3 ± 0.5°C |
| pH optimum for stability | 8.5 ± 0.5 | 8.5 ± 0.5 |

Tgi's for thermal denaturation were determined as described in Materials and Methods.

Fig. 1: Isolation of GPI-b from muscle. Preparative isoelectric focusing. The GPI from the DEAE Biogel column was concentrated, dialyzed, and run in a preparative isoelectric focusing column as described in Materials and Methods. The GPI protein from this column was run on a second isoelectric focusing column under identical conditions. The column was calibrated with the aid of a pI standard map (I to 10 fractions). The pI was measured at 10°C in 0.1M sodium phosphate buffer at pH 7.0 (pH 8). The isoelectric pH is indicated by the arrow. GPI activity (mg/g) was determined by the method described in Materials and Methods.

Fig. 2: The effect of pH on stability of GPI from Fusobacterium heteroclitum. Homogenates of muscle and liver tissues were inactivated at pH 5.0 to 7.0. Enzyme activity was determined in standard assay buffer at pH 8.0 and normalized to the highest value. Acetate buffer (100 mM) was used in the range of pH 5.0 to 6.0. Phosphate buffer (100 mM) in the pH range of 6.0 to 8.0. Dithiothreitol (10 mM) was used at pH 6.0 to 7.0. (pH) GPI-b (GPI-a).

Fig. 3: Linear plot showing competitive inhibition of 6-phenolphosphate. Data were collected at pH 7.0, 25°C. The levels of 6-phenolphosphate used were: 12.1 µM (<pH), 19.3 µM (<pH), 25.6 µM (<pH), 35.0 µM (<pH), 48.4 µM (<pH).