The Functional Impact of *Pgm* Amino Acid Polymorphism on Glycogen Content in *Drosophila melanogaster*

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ABSTRACT

Earlier studies of the common PGM allozymes in *Drosophila melanogaster* reported no *in vitro* activity differences. However, our study of nucleotide variation observed that PGM allozymes are a heterogeneous mixture of amino acid polymorphisms. In this study, we analyze 10 PGM protein haplotypes with respect to PGM activity, thermostability, and adult glycogen content. We find a twofold difference in activity among PGM protein haplotypes that is associated with a threefold difference in glycogen content. The latitudinal clines for several *Pgm* amino acid polymorphisms show that high PGM activity, and apparently higher flux to glycogen synthesis, parallel the low activity clines at G6PD for reduced pentose shunt flux in northern latitudes. This suggests that amino acid polymorphism is under selection at this branch point and may be favored for increased metabolic storage associated with stress resistance and adaptation to temperate regions.

A long-standing question in evolutionary genetics concerns the extent to which naturally occurring amino acid polymorphisms are associated with significant physiological variation (Watt 1994; Mitton 1998; Eanes 1999). Because physiological variation is probably influenced by alleles at many underlying loci, the effect of a single enzyme on such quantitative characters might be expected to be undetectable. The theory of metabolic flux through biochemical pathways originally posited that enzyme polymorphisms would have very little potential to alter pathway flux (Kacser and Burns 1973, 1981; Keightley 1989). This theory also predicts that substantial differences in enzyme kinetic properties are necessary to generate even detectable differences in flux and that most activity variation will be selectively neutral (Hartl *et al.* 1985). However, several studies present compelling evidence for selection on allozyme polymorphisms, notably PGI in Colias butterflies (Watt 1983; Watt *et al.* 1983, 1985); LDH in the killifish, *Fundulus heteroclitus* (Powers *et al.* 1991, 1993); and LAP in the blue mussel, *Mytilus edulis* (Koehn 1978; Koehn *et al.* 1980). In addition, chemostat studies with *Escherichia coli* suggest enzyme polymorphisms can regulate metabolic flux when the genetic background is manipulated (Dykhuizen *et al.* 1987; Dykhuizen and Dean 1990).

The most convincing evidence for selection on enzyme polymorphisms comes from patterns of nucleotide sequence variation and latitudinal clines in metabolic enzymes of *Drosophila melanogaster* (Kreitman and Akashi 1995; Eanes 1999). While several studies unsuccessfully attempted to couple the kinetic properties of metabolic enzymes with physiological traits that are potentially correlated with these enzymes' biochemical pathways (Middleton and Kacser 1983; Laurie-Ahlberg *et al.* 1985; Connors and Curtsinger 1986), other studies indicate that metabolic enzyme polymorphisms can influence pathway flux and represent adaptive protein variation (Eanes and Hey 1986; Labate and Eanes 1992; Freriksen *et al.* 1991, 1994). These studies predict that enzyme polymorphisms at branching pathways will be the targets of natural selection because they can potentially alter flux.

Phosphoglucomutase (PGM; EC 2.7.5.1) resides at the glycolytic pathway branch leading to glycogen synthesis. Variation at this step could in principle contribute to the regulation of carbohydrate storage through the breakdown or synthesis of glycogen (Ray and Roscelli 1964; Hirose *et al.* 1970). Interestingly, PGM is highly polymorphic for allozymes in many organisms (Dawson and Jaeger 1970) and has been the focus of functional studies in *D. melanogaster* (Fucci *et al.* 1979), the sea anemone *Metridium senile* (Hoffmann 1985), the oyster *Crassostrea gigas* (Pogson 1989, 1991), and Colias butterflies (Carter and Watt 1988). Several studies show a strong positive correlation between PGM activity and glycogen biosynthesis that is dependent on diet (Flisinska-Bojanowska *et al.* 1994; Guedon *et al.* 2000). Glycogen content in field mice is highly dependent on PGM genotype after restricted food supply (Leigh Brown 1977), and PGM deficiencies result in glycogen-storage disease in humans (Sugie *et al.* 1988) and cause starchless mutants in plants (Hanson and McHale 1988). Although line-specific PGM activity vari-

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ation is correlated with glycogen content in *D. melanogaster* (Clark and Keith 1988; Clark 1989), the contribution of allelic variation at the *Pgm* locus is unclear (Fucci *et al.* 1979; Carfagna *et al.* 1980; Pontecorvo *et al.* 1986).

Although earlier work found no evidence of geographic variation for PGM allozymes in *D. melanogaster* (Oakeshott *et al.* 1981), our recent study of sequence variation at *Pgm* found that in addition to the amino acid polymorphisms responsible for the three common allozyme alleles (*Medium*, *Fast*, and *Slow*), many electrophoretically cryptic, but common, amino acid polymorphisms segregate within the *Medium* allozyme allele (Verrelli and Eanes 2000). Our study reveals latitudinal clines for specific *Fast* and *Slow* allozyme alleles in addition to a single common *Medium* haplotype, which is the combination of two amino acid polymorphisms (Verrelli and Eanes 2001). The extensive amino acid variation and the strong latitudinal clines support the presence of adaptive PGM variation. Previous analyses of functional differences and selection of allozymes at this locus in *D. melanogaster* may have been obscured by the underlying amino acid variation of the allozyme alleles. Because enzymes at pathway branch points may represent the best candidates for regulating metabolic flux (LaPorte *et al.* 1984; Keightley and Kacser 1987; Keightley 1989), it is of interest to determine whether PGM protein variation alters flux into glycogen synthesis. In this report, we examine the activity and thermostability of *Pgm* alleles bearing different amino acid polymorphisms and relate these differences to glycogen content.

MATERIALS AND METHODS

Fly samples: The genetic lines analyzed in this study are a subsample of 500 extracted nonlethal third chromosomes from isofemale lines established from 10 populations collected in the eastern United States in fall 1997. The 10 PGM protein haplotypes in Table 1 account for ~95% of the overall PGM protein haplotype diversity and represent nine amino acid polymorphisms found repeatedly in our sample of extracted third chromosomes (Verrelli and Eanes 2001). We were able to recover at least 10 independent copies of each protein haplotype from the sample of 500 extracted chromosomes.

Lines were maintained at 25° on standard cornmeal medium in 8-dram vials. Table 2 shows the number of independent lines (n) sampled for each of the 10 protein haplotypes. After 5 days of egg laying, adults (four to five pairs) were purged from vials. Emerging adults were collected between 5 and 7 days posteclosion, transferred to fresh media, aged an additional 5 days, and then rapidly frozen at −80°. Although females show greater enzyme activity and glycogen content than males on average, enzyme activities were highly correlated between the two sexes, and because much of the assay preparation is labor intensive, we measured only females to generate a larger sample size. Five flies were homogenized with a motorized grinder in buffer (0.01 m KH_2PO_4, 1.0 mm EDTA, pH 7.4) of a total volume of 1 ml. All homogenates were prepared in six randomized blocks of 18 and were centrifuged at 10,000 rpm for 1.5 min at 4°. The supernatant was removed and immediately placed on ice. All activity assays (including thermostability measures) were completed in the same day in a randomized block design and remaining homogenate was then frozen at −80°. These were thawed and glycogen and protein assays were completed in a randomized block design on the following day. Our initial pilot studies with randomly drawn homogenates showed no effects on the activity, glycogen, or protein measures after extended periods of freezing at −80°.

Activity measurements: Assays for PGM activity were carried out on a Beckman (Fullerton, CA) DU 640 UV/visible spectrophotometer at 25°. The reagent mix contained 0.83 mm glucose-1-phosphate, 0.5 mm NADP, 1.0 mm $MgCl_2$, 3.1 units/ml G6PD in 20 mm Tris-HCl (pH 7.4). The assay contained 425 μl of this reagent and 25 μl of fly homogenate. This reaction was followed at OD_{340} (Stam and Laurie-Ahlberg 1982), and initial rates were determined from change in OD every 12 sec measured over the initial 3 min with controls run in tandem with each assayed block. PGM activity is expressed as units (micromoles of NADP reduced, per minute) per milligram of soluble protein.

Glycogen measurements: This standard procedure measures free glucose from hydrolyzed glycogen and is available from Sigma Biochemical (St. Louis) as kit 510A. One powder cap of glucose oxidase and peroxidase (PGO enzyme) is dissolved in 100 ml of dH_2O, with 1.6 ml of *o*-dianisidine dihydrochloride (50 mg/20 ml), 10 units of amyloglucosidase (Sigma A-3514), and placed on ice. The assay contained 450 μl of this reagent and 50 μl of fly homogenate and was incubated in a 37° water bath for 30 min before immediate transfer to ice. These assays were measured at OD_{450} and glycogen concentration was determined from glycogen standards (Sigma G-0885). Concentrations were expressed as milligrams of glycogen per milligram of soluble protein.

Thermostability measurements: To estimate haplotype-specific enzyme thermostabilities, PGM activity was measured after variable periods of time at 50°. Four replicate copies for each of the 10 PGM protein haplotypes were assayed as follows. For each replicate copy, 10 aliquots of 25 μl fly homogenate were placed in a 50° water bath and at 1-min intervals (up to 10 min) a single aliquot was removed and immediately transferred to ice. Aliquots of 25 μl were kept on ice and served as controls for each time interval. The proportion of enzyme activity remaining after heat treatment was compared to a control. As in Hall (1985), the decline in enzyme activity with time was treated as a first-order exponential decay process and denaturation constants (k_D) were determined using the relationship

$$(E/E^v)_t = e^{-k_D t},$$

where (E/E^v) is the proportion of initial enzyme activity remaining at time t. The slope of the line from the linear regression of $\ln(E/E^v)$ on time results in an estimate of k_D. A mean k_D was calculated from four replicate copies per PGM protein haplotype.

Soluble protein measurements: The soluble protein measurements were conducted using a protein assay from Bio-Rad (Hercules, CA) kit no. 500-0006. This dye solution is diluted with 4 volumes of dH_2O and paper filtered. The assay contained 1 ml of this reagent plus 20 μl of fly homogenate and was incubated at room temperature for 5 min. Reactions were measured at OD_{595} and total soluble protein concentration was determined from bovine serum albumin standards (Sigma A-2153). All assays were standardized by soluble protein to compare activity and glycogen measures. Soluble protein is used as a covariate because differences in mass or body size may not be strictly correlated with overall protein (Clark and Keith 1988). However, we find no evidence for body size

TABLE 1

List of *Pgm* amino acid haplotypes

Nucleotide position:	25	178	226	1324	1340	1626	1998	2055	2259
Residue[a]:	9^S	36	52	240^F	245	341	465	484	530
Haplotype	A	T	V	R	E	V	T	V	A
1	—	—	A	—	—	—	—	—	—
2	—	—	A	—	—	—	S	—	—
3	—	—	—	—	—	—	—	—	—
4	—	—	A	—	—	—	—	L	—
5	—	—	A	L	D	—	—	L	—
6	—	M	—	—	—	—	—	L	—
7	—	—	—	—	—	—	—	L	—
8	T	—	A	—	—	—	—	—	—
9	—	—	A	—	—	M	—	—	—
10	—	—	A	—	—	—	—	L	T

^{S} and ^{F}, substitutions causing *Slow* and *Fast* allozyme alleles, respectively.
[a] Refers to amino acid residue.

differences across our extracted third chromosome lines, and our analysis in this study shows no effect of soluble protein on enzyme activity or glycogen content.

Statistical analyses: Although the genetic backgrounds are randomized by repeated crosses with the *TM3/TM6* balancer stock, third chromosomes remain intact. For each PGM protein haplotype, replicates are segregating in different isolated third chromosome backgrounds; however, it was necessary to sample these replicates from different localities along the latitudinal cline because several protein haplotypes have low overall frequencies. Therefore, variation could simply reflect third chromosome background effects in different populations. Population sample was treated as a variable to factor out any potential population effect and all measured variables (*e.g.*, glycogen, activity, etc.) were analyzed with respect to PGM protein haplotype.

This investigation was designed to answer three questions concerning the contribution of *Pgm* amino acid polymorphisms to enzyme activity and thermostability and their relationship to glycogen content. First, we were interested in the amino acid polymorphisms at nucleotide positions 226 (Val to Ala at residue 52; V52A) and 2055 (Val to Leu at residue 484; V484L). Our previous study found that the V52A and V484L amino acid polymorphisms are the most common in natural populations and that combinations of these two polymorphisms show the strongest and steepest protein haplotype clines (Verrelli and Eanes 2001). Therefore, we first analyzed the four protein haplotypes composed of only these two amino acid polymorphisms (haplotypes 1, 3, 4, and 7 in Table 1) for enzyme activity and glycogen content in a two-factor ANOVA. By treating polymorphisms V52A and V484L as independent variables, we can determine the relative contribution of these two polymorphisms to both enzyme activity and glycogen content.

Second, we were interested in whether additional *Pgm* amino acid polymorphisms contribute to differences in activity, thermostability, and glycogen content. Our previous statistical analysis of these less frequent amino acid polymorphisms shows that they are in strong linkage disequilibrium with the common V52A and V484L amino acid polymorphisms and are likely hitchhiking along with these two polymorphisms (Verrelli and Eanes 2001). However, we were interested in determining whether they have added functional and structural effects. Therefore, we compared all 10 PGM protein haplotypes in Table 1 for each of the activity, thermostability, and glycogen content measures by *a posteriori* Bonferroni multiple range tests (Sokal and Rohlf 1995) to determine if there are significant protein haplotype outliers that may represent potential adaptive variation. Finally, while there is likely heterogeneity among both activity and glycogen, we were particularly interested in the correlation between these two traits. Therefore, we performed a nonparametric test to determine this association (Sokal and Rohlf 1995).

RESULTS

Table 2 summarizes enzyme activity, thermostability, and glycogen content for the 10 PGM protein haplotypes listed in Table 1. The protein haplotype numbers are consistent with those used in Verrelli and Eanes (2001). The range in enzyme activity spans twofold and the range in glycogen content is greater than threefold. This variation is first addressed with respect to the four major protein haplotypes associated with the V52A and V484L amino acid polymorphisms and then with respect to additional protein haplotypes that are derived subsets of these four major protein haplotypes.

Figure 1 presents the activity and glycogen data for the polymorphisms at amino acid residues 52 and 484 and represents PGM protein haplotypes 1, 3, 4, and 7 in Tables 1 and 2. An analysis of variance finds significant enzyme activity variation contributed by both the V52A and V484L polymorphisms ($F_s = 14.5$ and 10.1, respectively; $P < 0.001$). There is also a highly significant interaction between polymorphisms ($F_s = 9.9$; $P < 0.001$). This interaction is clearly demonstrated by the large difference in enzyme activity for the protein haplotype Ala52/Val484 in Figure 1. Additive variation at the two residues explains 13% of the overall variation in

TABLE 2

***Pgm* haplotype means and standard errors for enzyme activity, glycogen, and thermostability**

Haplotype	*n*	*Pgm* activity[a]	Glycogen[b]	*n*	Thermostability[c]
1	12	3.32 ± 0.198	2.45 ± 0.287	4	0.18 ± 0.023
2	10	2.37 ± 0.097	1.63 ± 0.275	4	0.17 ± 0.027
3	12	2.19 ± 0.164	1.55 ± 0.220	4	0.16 ± 0.035
4	12	2.07 ± 0.150	1.09 ± 0.260	4	0.31 ± 0.068
5	10	1.89 ± 0.178	0.72 ± 0.176	4	0.80 ± 0.061
6	10	1.75 ± 0.091	0.78 ± 0.258	4	0.52 ± 0.071
7	12	2.05 ± 0.151	1.21 ± 0.196	4	0.13 ± 0.033
8	10	3.06 ± 0.170	2.25 ± 0.282	4	0.21 ± 0.022
9	10	1.97 ± 0.180	1.62 ± 0.265	4	0.55 ± 0.068
10	10	1.85 ± 0.155	1.68 ± 0.213	4	0.28 ± 0.035

[a] Units per milligram of soluble protein.
[b] Milligrams glycogen per milligram of soluble protein.
[c] Absolute value of k_D; high values indicate low thermostability (see text).

enzyme activity, while 35% is explained by the interaction.

Figure 1 also shows the same pattern for glycogen content. Glycogen content differs significantly between substitutions at residue 484 ($F_s = 7.2$; $P < 0.05$); however, like activity, glycogen is highly dependent on the combination of polymorphisms at residues 52 and 484 ($F_s = 5.2$; $P < 0.05$). This interaction explains almost 25% of the overall glycogen variation, while the two independent residues explain only 15%. As with activity, protein haplotype Ala52/Val484 shows the highest glycogen content.

The Ala52/Val484 protein haplotype 1 possesses the highest enzyme activity and glycogen content of all 10 PGM protein haplotypes (Table 2). After the data were log transformed to normalize the means, a Bonferroni multiple comparisons test shows that haplotypes 1 and 8 differ significantly from all other protein haplotypes in enzyme activity. Haplotype 8 is apparently derived from haplotype 1 and is the common *Slow* allozyme allele (see Table 1). The same test of the means for glycogen content shows that haplotypes 1 and 8 are significantly greater than haplotypes 5 and 6, which have the lowest values of all 10 protein haplotypes for glycogen content.

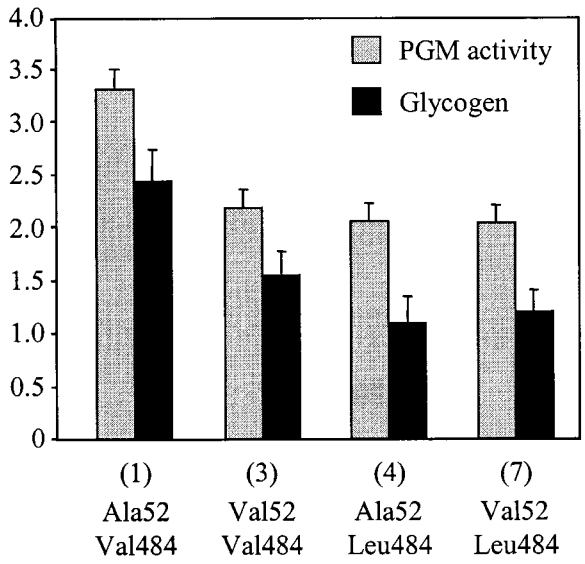

FIGURE 1.—Means ± SE of PGM activity (micromoles of NADP reduced, per minute per milligram of soluble protein) and glycogen content (milligrams of glycogen per milligram of soluble protein) for protein haplotypes composed of only the common V52A and V484L amino acid polymorphisms. Numbers in parentheses refer to the protein haplotypes in Table 1.

Figure 2 plots the glycogen content and mean enzyme activity for all 10 PGM protein haplotypes. There is comparatively high enzyme activity and glycogen content for haplotypes 1 and 8. As previously mentioned, haplotypes 5 and 6 possess both low enzyme activity and low glycogen content. A nonparametric test finds that, overall, glycogen content is significantly associated with enzyme activity (Kendall's $\tau = 0.512$; $P < 0.05$; SOKAL and ROHLF 1995).

Finally, Figure 3 plots the relationship between thermostability and enzyme activity. Larger k_D values indicate greater sensitivity to thermal degradation after extended periods at 50°. Preliminary experiments at lower temperatures found no differences in stability among haplotypes after 15 min; however, many alleles lost complete PGM activity after just 2 min at 55°. We chose 50° because this temperature represented the range most likely to demonstrate differences in protein stability among PGM protein haplotypes. Although these protein haplotypes represent a broad range of enzyme activity, there is very little difference in thermostability among them. One notable observation from Figure 3 is that two of three protein haplotypes (haplotypes 5 and 6) with low thermostability also possess low activity. However, overall it is apparent that PGM enzyme activity is not associated with thermostability.

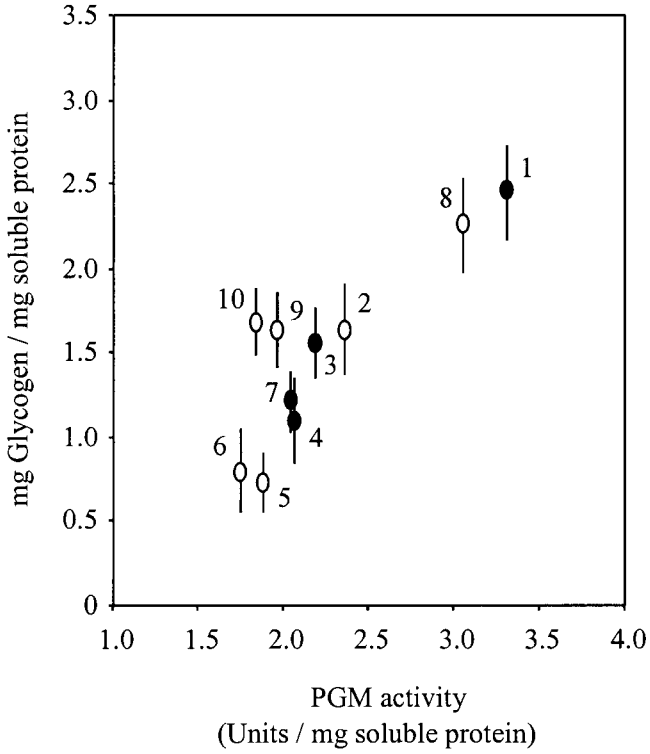

FIGURE 2.—Relationship between haplotype mean PGM activity and glycogen content (means ± SE). Solid data points refer to the four protein haplotypes that are composed of only the common V52A and V484L amino acid polymorphisms.

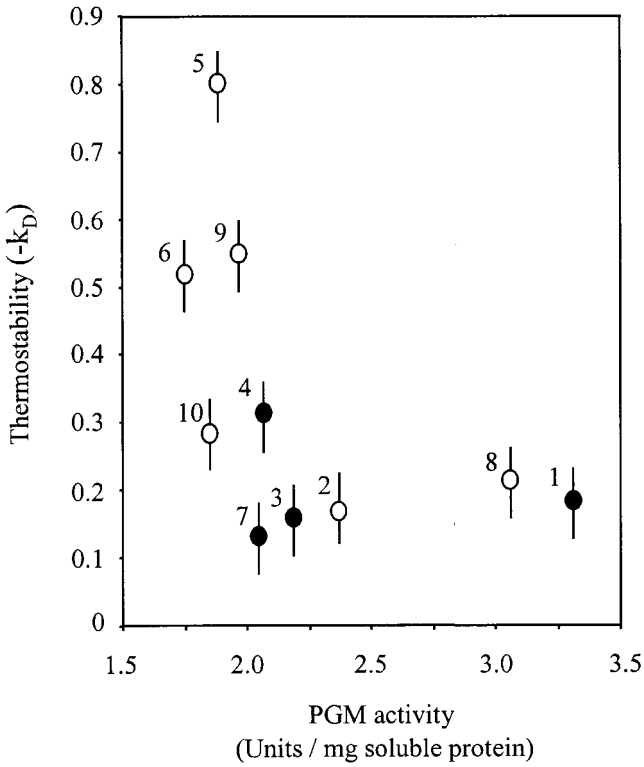

FIGURE 3.—Relationship between haplotype mean PGM activity and thermostability (means ± SE). Solid data points refer to the four protein haplotypes that are composed of only the common V52A and V484L amino acid polymorphisms. Larger ($-k_D$) values indicate lower thermostability.

DISCUSSION

This is the first study to demonstrate functional differences between protein variants at the *Pgm* locus in *D. melanogaster* and shows that *Pgm* is a quantitative trait locus for glycogen content. Although an earlier investigation found no allele-specific characteristics at this locus (FUCCI *et al.* 1979), this previous study classified *Pgm* alleles by allozyme mobility (*i.e.*, compared *Medium* and *Slow* allozymes). When unambiguously defined by their amino acid mutations, PGM protein haplotypes show a twofold difference in activity, measured here as V_{max}. Of even greater significance is that glycogen content is highly correlated with PGM enzyme activity. This observation has implications for the role of enzyme variation in regulating flux and metabolic energy pools. With this in mind, what can we predict about the connection between *Pgm* amino acid polymorphism and its functional impact in natural populations?

Enzyme activity and glycogen content: With 21 amino acid polymorphisms discovered at *Pgm* in *D. melanogaster* (VERRELLI and EANES 2000, 2001), this study investigates only a fraction of the overall variation segregating in natural populations. However, many of these amino acid polymorphisms are infrequent and there is strong linkage disequilibrium across the entire gene (VERRELLI and EANES 2001). Although this study investigates only 9 amino acid polymorphisms, the 10 protein haplotypes represent ~95% of the protein haplotype diversity at *Pgm*. This indicates that the functional differences found in this study are not generated by rare mutations in natural populations that confer unusually high or low enzyme activities.

It is possible that most *Pgm* amino acid mutations decrease enzyme activity and are therefore deviations from "optimal" protein function (KNOWLES 1991; KUHLMAN and BAKER 2000). This would predict that the ancestral protein haplotype possesses the highest enzyme activity. The *Pgm* sequence from *D. simulans* indicates that the Val52/Val484 haplotype 3 is the ancestral amino acid sequence (VERRELLI and EANES 2000). Therefore, it is obvious that amino acid mutations to this ancestral protein haplotype resulted in derived protein haplotypes with both increased and decreased activity that segregate in natural populations.

This study uses homozygous third chromosomes; therefore, it is possible that additional variation segregating on this chromosome contributes to the variation in enzyme activity and glycogen content. While it is possible that the common and clinal inversion *In(3L)P* that is located in close proximity to *Pgm* (VERRELLI and EANES 2000) has some effect in natural populations, it is not present in our sample and, therefore, it cannot explain the functional differences found here. While

there are likely other factors that contribute to PGM activity and glycogen content variation among chromosomes (Laurie-Ahlberg *et al.* 1980; Wilton *et al.* 1982; Clark and Keith 1988; Clark 1989), these are effectively randomized within each protein haplotype, except for regions in potential linkage disequilibrium with *Pgm*. Each of the 10–12 replicates is an independently recovered copy of that PGM protein haplotype and all replicates segregate for different third chromosomal backgrounds. This does not preclude the possibility that a gene in strong linkage disequilibrium with *Pgm* contributes to the activity or glycogen variation. One would have to propose that a closely linked factor or modifier that increases glycogen content also increases PGM activity (or vice versa) or that high and low PGM activity alleles are in strong linkage disequilibrium with alleles at a second factor that affect glycogen content in the same magnitude. With either case, it appears that PGM activity is apparently an important predictor of glycogen content.

Clinal selection on PGM activity variation: We are particularly interested in the relationship between enzyme activity and geographic variation because several of the PGM protein haplotypes in this report exhibit strong latitudinal clines (Verrelli and Eanes 2001). Haplotype 1 is a derived allele and, with an average frequency of ~32%, it is the most frequent protein haplotype along the sampled latitudinal cline and it possesses epistatic activity effects from both the V52A and V484L polymorphisms. This protein haplotype also shows the greatest geographic variation, increasing from 20 to 84% with increasing latitude, and our statistical analysis indicates that linkage with it can explain all amino acid clines at this locus except that associated with haplotype 8 (*Slow* allozyme allele), which is also positively correlated with higher latitudes. The fact that haplotypes 1 and 8 show twice the PGM activity of all other haplotypes certainly suggests that increased PGM activity and consequently higher glycogen content may be favored in higher latitudes. The two haplotypes show similarly high enzyme activities and differ only by the A9T polymorphism on haplotype 8. Therefore, it is unclear whether this *Slow* allozyme allele possesses some additional advantage or if it simply behaves as another high activity Ala52/Val484 haplotype with no effect from the A9T polymorphism.

All other protein haplotypes comprise a statistically homogeneous subset with lower activity and glycogen content. However, of interest is the single *Fast* allozyme allele (haplotype 5), which shows a positive association with lower latitudes (Verrelli and Eanes 2001). While this cline can be explained as simply a consequence of other stronger protein haplotype clines, it is interesting to note that this haplotype possesses one of the lowest PGM activities, thermostabilities, and glycogen contents. Haplotypes 2 and 9 are both derived from haplotype 1; however, neither exhibits clinal variation and they also possess lower enzyme activity. Therefore, although they are Ala52/Val484 haplotypes, the added V341M and T465S polymorphisms apparently cause intermediate activity and possibly explain the lack of clinal variation for these two haplotypes.

Given the association between PGM protein haplotype and glycogen content and the strong latitudinal clines for PGM protein haplotypes, what does this predict about glycogen content in natural populations? From the observed PGM protein haplotype frequencies across the cline, we can compute the expected genotype frequencies for all 10 populations. Using the mean glycogen content associated with each of the 10 protein haplotypes in Table 1, and assuming a simple additive model (between protein haplotypes), we can estimate the mean glycogen content for all possible genotype combinations. From this we can propose population means for glycogen content. These calculations predict a significant correlation between predicted mean glycogen content and population latitude ($m = 0.029$, $r^2 = 0.765$; $P < 0.001$), but because haplotype 1 is the majority haplotype, shows a strong association with latitude, and possesses the highest glycogen content, this correlation is expected.

It is of interest to determine if the glycogen content predicted from PGM protein haplotype variation compares with glycogen content found in natural populations. Our data for mean glycogen content (milligrams of glycogen per milligrams of soluble protein) from a sample of isofemale lines from the same 10 populations along the latitudinal cline (L. M. Matzkin, B. C. Verrelli and W. F. Eanes, unpublished data) show a nonsignificant, yet positive, association with increasing latitude ($m = 0.022$, $r^2 = 0.309$; $P = 0.09$). It is possible that modifiers that affect PGM activity or glycogen content in natural populations are absent or are masked in the extracted lines, and this could explain the different pattern observed in our isofemale lines. Although this analysis shows there is no apparent cline in glycogen content *per se*, this slope is not significantly different from the slope predicted above ($F_s = 0.019$; $P > 0.90$), which implies that the PGM protein haplotype cline may potentially explain geographic variation in glycogen content.

Because temperature plays a large role in catalyzing enzymatic reactions, it is possible that the twofold increase in PGM activity reflects temperature compensation to maintain constant glycogen content. This suggests that the differences in enzyme activities are simply a response to a temperature gradient across the latitudinal cline. If selection favors *Pgm* amino acid polymorphism in maintaining uniform activity across the thermal cline, this might explain the weak association of glycogen content with latitude. Thermal compensation can also be accomplished by altering transcription levels to maintain enzyme activity (Crawford and Powers 1992; Segal *et al.* 1999); however, the functional differ-

ences found in this study are associated with specific amino acid polymorphisms. Whether these amino acid polymorphisms are under selection to maintain homeostasis or to alter enzyme function over the latitudinal cline, it is clear that *Pgm* amino acid polymorphism is significantly associated with enzyme activity and glycogen content.

Stability *vs.* activity: An issue in the evolution of protein structure concerns potential trade-offs between enzyme stability and activity (review by Somero 1995; Zavodszky *et al.* 1998; Spiller *et al.* 1999). It is argued that increased thermostability is achieved at the cost of decreased structural flexibility and this results in decreased enzyme activity. It is possible that PGM protein haplotypes have lower activity in southern latitudes because of the potential need for more thermostable alleles. Figure 3 shows that, with respect to thermostability and V_{max}, there is no apparent trade-off. Thermal regulation is more difficult for small insects compared to larger ectotherms; therefore, the ability to withstand differences in temperature may be enhanced by thermostable enzymes (Watt 1994; Dahlhoff and Rank 2000). It is also possible that thermostability is achieved by enhancing enzyme-substrate binding efficiency (Hochachka and Somero 1984), and, therefore, investigating the temperature effects on substrate affinity (K_m) may reveal more about the impact of amino acid polymorphism at *Pgm*.

Inferences from rabbit PGM three-dimensional structure: The three-dimensional (3D) structure for PGM is of interest because of the amino acid similarity in functional regions among phylogenetically distant taxa (Whitehouse *et al.* 1998; Levin *et al.* 1999). The 3D structure has been determined only from rabbit PGM (Dai *et al.* 1992; Liu *et al.* 1997), but because of the similarity in secondary structure among homologous proteins, it is a valuable template for exploring potential structural implications of PGM polymorphisms in *D. melanogaster*. As many as 50 amino acid residues from >30 different regions of the secondary structure converge to produce the highly conserved binding domains labeled on the PGM 3D structure shown in Figure 4 (Liu *et al.* 1997). After alignment of the *D. melanogaster* and rabbit PGM amino acid sequences, our analysis of 21 amino acid polymorphisms finds none are unequivocally in the predicted binding domains. One exception is a reported PGM null allele (Langley *et al.* 1981; Burkhart *et al.* 1984), which contains a single amino acid substitution (G109A, see Verrelli and Eanes 2000) that is seven residues from the highly conserved phosphoserine Ser116 in the active site (Liu *et al.* 1997) and confers almost no enzyme activity (our unpublished data).

We were particularly interested in the locations of several specific amino acid changes and these are labeled in Figure 4. The common V484L polymorphism is found in a region near the phosphate-binding loop

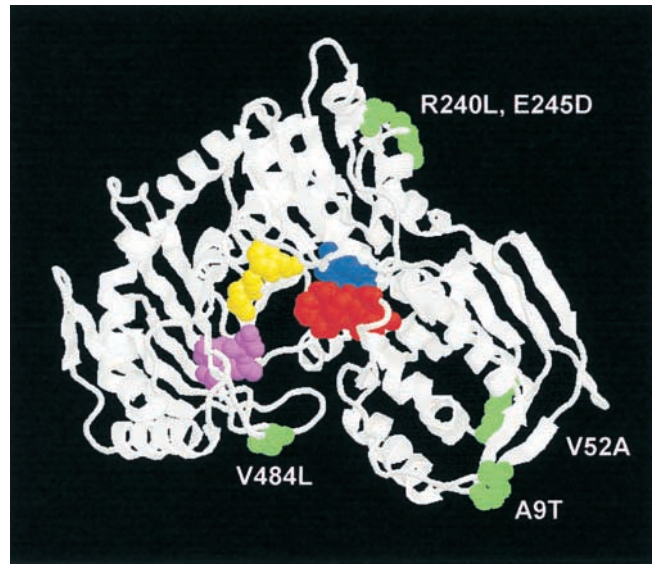

Figure 4.—Predicted location of five *D. melanogaster Pgm* amino acid polymorphisms (green) on the rabbit PGM three-dimensional protein structure. Active site is composed of four binding loops: phosphate-transfer loop (red), Mg^{2+} binding loop (blue), glucose binding loop (yellow), and phosphate-binding loop (purple).

that is highly conserved across many diverse taxa (Levin *et al.* 1999). This conserved region forms a loop positioned next to the active site crevice and this may explain the reduction in activity associated with this polymorphism. The *Fast* allozyme allele (haplotype 5) has the V484L polymorphism in addition to two close amino acid polymorphisms (the charge change R240L and E245D), which appear to alter a conserved hydrophobic region and are predicted to lie on the outer protein surface behind the Mg^{2+} binding site. Charge changes from hydrophobic to hydrophilic residues on the protein surface disrupt α-helix structures and subsequent protein folding (Argos *et al.* 1979; Menendez-Arias and Argos 1989), and this may explain both the lower activity and thermostability for haplotype 5.

The common V52A (haplotype 1) and A9T (*Slow* allozyme, haplotype 8) polymorphisms are near a well-conserved region behind the phosphate-transfer loop of the active site domain. Although the A9T polymorphism confers a *Slow* allozyme mobility, this mutation alone does not predict a change in net charge. Many charged residues in this structural domain form bonds with the phosphoserine Ser116 in the active site (Liu *et al.* 1997), and it is possible that the A9T polymorphism alters the positioning of these buried charged residues, which results in an indirect charge change (Dill 1990). Although these observations are based on a predicted 3D structure and are limited, as the number of known protein structures increases, this comparative approach reveals another level of resolution in enhancing understanding of enzyme evolution.

Finally, the high activity and glycogen content repre-

sentative of the Ala52/Val484 haplotype 1 appear to involve an interaction between the V52A and V484L polymorphisms. A first expectation would be that this involves a physical interaction between these two residues, but this is not apparent in the predicted PGM structure where the residues are in different domains. This does not preclude the possibility of a distant structural interaction but suggests some other mechanism. The two nucleotide polymorphisms, or an additional site outside of *Pgm* in close linkage disequilibrium, could regulate translation or gene expression by altering mRNA secondary structure as suggested in the complex case of *Adh* (Laurie and Stam 1994). It is difficult to evoke a compensatory substitution model (Kirby *et al.* 1995; Parsch *et al.* 1997) since these two mutations show no linkage disequilibrium (Verrelli and Eanes 2001). However, examining regions outside the *Pgm* gene and measuring PGM protein mRNA levels could elucidate the role these amino acid mutations play in regulating enzyme activity.

Control of flux and glycogen synthesis: The strong association between activity and glycogen content indicates that *Pgm* amino acid polymorphism can significantly alter flux in the pathway to glycogen synthesis. As a consequence, these polymorphisms could be favored through selection on glycogen content. Our study of life history variation finds increased life span, lipid storage, and starvation resistance in northern latitudes and we posit this is an adaptive response to the need for adults to overwinter in temperate regions (L. M. Matzkin, B. C. Verrelli and W. F. Eanes, unpublished data). It is possible that glycogen storage may be causally connected to starvation resistance as an energy reserve (Chippindale *et al.* 1996, 1998) and higher PGM activity may simply facilitate glycogen turnover in response to lowered nutrient levels (Leigh Brown 1977). Alternatively, because glycogen can account for ~30% of the body mass for *D. melanogaster* (our unpublished data), there may be trade-offs associated with body weight. For example, tropical regions may create an environment in which flies can remain active almost year round, and lower body mass (and subsequently lower glycogen content) may be favored; however, this selection pressure may be relaxed in temperate regions where potentially long periods of inactive overwintering and diapause may allow increased glycogen storage. Additionally, because flight performance reduces with decreasing temperature (Lehmann 1999), increased glycogen storage and utilization may provide a power reserve for more efficient flight in temperate regions.

While the extensive amino acid polymorphism and clinal variation made a significant case for natural selection acting at the *Pgm* locus (Verrelli and Eanes 2000, 2001), the functional basis for this selection is established by our observations on enzyme activity and glycogen variation. In addition to *Pgm*, both *G6pd* (Oakeshott *et al.* 1983) and *Hex* (Duvernell and Eanes 2000) show strong amino acid polymorphism clines with latitude, and all three enzymes share glucose-6-phosphate at the head of the glycolytic pathway. The PGM protein haplotype cline predicts high activity and glycogen content in higher latitudes and parallels the G6PD low activity allele cline that clearly reduces pentose shunt flux (Eanes *et al.* 1990; Labate and Eanes 1992). This suggests that *Pgm* amino acid polymorphism may be favored to redirect flux away from the main pathway and the pentose shunt and into glycogen synthesis in northern latitudes and the G6PD polymorphism is an associated response to selection for glycogen content. Although Kacser and Burns' (1973, 1981) theory had initially predicted that metabolic flux will be insensitive to enzyme variation, others had predicted that pathway branch points may demonstrate the ability to control metabolic flux (LaPorte *et al.* 1984; Keightley and Kacser 1987; Keightley 1989). Despite the positive association between PGM activity level and glycogen content observed here, it is not clear that this results from increased flux through PGM. The reaction is freely reversible and the equilibrium is thermodynamically strongly shifted toward glucose-6-phosphate. One possibility is that in competition with the other branches, the glucose-6-phosphate concentration is shifted to stimulate or inhibit the activity of other steps such as glycogen synthase, trehalose-6-phosphate synthase, glucose-6-phosphatase, or hexokinase. The latter two enzymes are reported to exert significant flux control over glyconeogenesis in transgenic rats (O'Doherty *et al.* 1996; Trinh *et al.* 1998). Irrespective of mechanism, it is apparent from the observations on PGM, as well as other carefully described enzyme systems, that protein polymorphism in natural populations can modulate fluxes and thus come under selection (Eanes 1999).

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