Do "neutral" protein positions really exist? A case study with allostery in human liver pyruvate kinase

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Abstract

In the goal of interpreting human exomes, when predictive programs assign functional importance to some positions, they implicitly assume the existence of non-important positions – those that accommodate many side chain chemistries without altering function ("neutral"). However, very few (if any) experimental studies have demonstrated the existence of neutral positions. We sought experimental evidence for neutral positions using human liver pyruvate kinase (hLPYK) as a model system. To that end, we used multiple evolutionary criteria to identify 20 possibly neutral positions. Nine positions were further tested with a total of 117 amino acid substitutions. Although "all" potential hLPYK functions can never be explored, we measured effects on 5 parameters associated with substrate/ligand affinities and allosteric coupling. At each position, the aggregate outcomes of multiple variants were used to quantify "neutrality" scores. Three of the nine positions showed perfect neutral scores in all 5 parameters; a fourth position had high neutral scores. Although our strategy for predicting positions had low predictive power for the identification of neutral positions, all positions had neutral scores that were much higher than positions in functional sites. Given this evidence for the existence of neutral positions, similar studies should be carried out for other proteins to generate a database of well characterized neutral positions that can then be available to benchmark and validate predictions about amino acid substitutions.

Keywords: Neutral position, allosteric regulation, pyruvate kinase, exome interpretation, mutation, predicting function

Introduction

Attempts to evaluate which residues in a protein contribute to function are often initiated with a sequence alignment including many homologs of a protein family. In this approach, conserved positions are considered to have some functional importance, resulting in the observed evolutionary conservation. The corollary to considering one type of position to have function is an implied lack of function at other positions: neutral positions. Although assumed, very few neutral positions have been reported in the literature. More recent co-evolution (Brown & Brown, 2010; Dekker, Fodor, Aldrich, & Yellen, 2004; Fodor & Aldrich, 2004; Gobel, Sander, Schneider, & Valencia, 1994; Kass & Horovitz, 2002; Olmea, Rost, & Valencia, 1999; Olmea & Valencia, 1997) and evolutionary trace (Gu & Vander Velden, 2002; Gu et al., 2013; Lichtarge, Bourne, & Cohen, 1996; Mihalek, Res, & Lichtarge, 2006; Ye, Vriend, & Ijzerman, 2008) methods of evaluating families of proteins predict function for residues that are not completely conserved throughout a protein family. As more analyses predict function to less conserved positions, we asked if neutral positions truly exist. Therefore, the goal of this study was to look for evidence that neutral positions exist.

The all-encompassing nature of "protein function" presents a challenge to these studies. We can never confirm that *all* possible functions have been tested. Therefore, the approach we employed here was to use a semi-high throughput technique that could report on multiple functions in one assay. The protein used in this study is human liver pyruvate kinase (hLPYK), the enzyme that catalyzes the final reaction in glycolysis in the liver. The affinity of hLPYK for its substrate, phosphoenolpyruvate (PEP), is modified when the protein binds allosteric effectors. Alanine is an allosteric inhibitor, reducing PEP affinity. Fructose-1,6-bisphosphate (Fru-1,6-BP) is an allosteric activator, increasing PEP affinity. We evaluated both allosteric activation and allosteric inhibition

of hLPYK and thereby characterized five parameters for each variant protein (Table 1). By considering multiple functional parameters, we increased the probability of detecting functional consequences due to amino acid substitution. Nonetheless, due to the nature of the question, this study only provides evidence that is consistent with some positions being neutral.

Our results for 117 variants at 9 hLPYK positions illuminates trends that are consistent with the existence of neutral positions. However, identification of the tested positions used multiple filters before experimental testing, and several of the tested positions were not perfectly neutral. This suggests that current algorithms over-estimate the prevalence of neutrality among protein positions and substitutions.

Materials and Methods

The GenBank RefSeq for the PKLR gene is NM_000298.5. Amino acid sequencing follows the HGVS rules, including using the methionine coded for by the ATG translation initiation codon as residue 1. Unless otherwise stated, all position numbers in this manuscript correspond to that of the human liver isoform.

To choose a tractable number of potentially neutral positions for experimental testing, we used available evolutionary and experimental information. We first used the PYK sequence alignment of Pendergrass *et al.* (Pendergrass, Williams, Blair, & Fenton, 2006) to identify positions with the least evidence for evolutionary constraints. To that end, we calculated the TEA-O (Ye et al., 2008) conservation and specificity scores and used co-evolution utilities to calculate pairwise co-evolutionary scores from four different algorithms (McBASC,(Gobel et al., 1994; Olmea et al., 1999; Olmea & Valencia, 1997) OMES, (Fodor & Aldrich, 2004; Kass & Horovitz, 2002) ELSC,(Dekker et al., 2004) and ZNMI(Brown & Brown, 2010)). Since each algorithm utilized a different scoring scale, we used their maximum and minimum scores to re-scale all scores from 0.01 (least patterned) to 1 (most patterned). The six standardized scores were then multiplied to generate a single composite score.

Using the rank-ordered list, the least patterned positions were further assessed using SNAP(Yana Bromberg, Yachdav, & Rost, 2008) to predict neutral/non-neutral outcomes for all 19 possible substitutions at each position. (SNAP makes predictions for individual substitutions rather than for positions.) Twenty of the top 22 positions had at most one non-neutral prediction, and the single non-neutral substitutions in positions 75, 138, 210, 388 and 470 was either a proline or tryptophan. (Our data below shows that position 138 accommodates tryptophan with no change in function, therefore at least that prediction was wrong.) Since proline has large effects on the

backbone and tryptophan inserts a very large side chain, we considered the aggregate prediction for the position was neutral. Two positions (59 and 248) were predicted to have multiple non-neutral substitutions and were further excluded. As described in Results, the final list of positions was chosen after comparison to existing experimental data.

For experimental assays, the creation of amino acid substitutions, protein expression, preparation and data analyses were the same as previously reported (Ishwar, Tang, & Fenton, 2015; Tang, Alontaga, Holyoak, & Fenton, 2017; Tang & Fenton, 2017). In brief, variant hLPYK proteins were created by mutating the coding region of the pLCII plasmid (originally a kind gift from Drs. Andrea Mattevi and Giovanna Valentini(Valentini et al., 2002)) using QuikChange (Stratagene) and primers designed to individually obtain all 19 substitutions at each position. After QuikChange mutagenesis, a single colony from each mutagenic reaction was sequenced. Although we did not obtain all 19 substitutions, this method generated a range of substitutions at each position in a cost-effective manner, without introducing a human bias into the final set of substitutions.

Next, wildtype and mutated genes were expressed in FF50 *E. coli* that lack the two *E. coli pyk* genes (Fenton & Hutchinson, 2009). Proteins were purified using ammonium sulfate fractionations and extensive dialysis (Ishwar et al., 2015; Tang et al., 2017; Tang & Fenton, 2017). Because this is a partial purification, accurate estimates of hLPYK concentrations were not possible and therefore k_{cat} values were not determined. Enzymatic activity was measured in a 96 well plate format using a lactate dehydrogenase/NADH coupled assay to monitor the change in A_{340} at different substrate PEP concentration. Due to the reliance of our assay on monitoring activity to first evaluate PEP affinity, no other parameters could evaluated for variants that lacked catalytic activity. For all active variants, the PEP concentration that was equal to ½ maximal

activity was used as $K_{app\text{-}PEP}$. This $K_{app\text{-}PEP}$ value was simultaneously determined over a concentration range of each allosteric (Ala and Fru-1,6-BP). For each ligand, the $K_{app\text{-}PEP}$ response to concentration was fit to:

$$K_{\text{app-PEP}} = K_{\text{a}}\left(\frac{K_{\text{ix}} + [\text{Effector}]}{K_{\text{ix}} + Q_{\text{ax}}[\text{Effector}]}\right), \qquad \text{Equation 1}$$

where K_a is the apparent affinity for ligand A (*e.g.*, substrate $K_{a\text{-}PEP}$) in the absence of ligand X (Ala or Fru-1,6-BP); $K_{a/x}$ is the apparent affinity for ligand A in the presence of saturating ligand X; K_{ix} is the affinity for ligand X (*e.g.*, $K_{ix\text{-}Ala}$ and $K_{ix\text{-}FBP}$) in the absence of ligand A; and $K_{ix/a}$ is the affinity of the protein for ligand X in the presence of saturating ligand A (Table 1). In equation 1, Q_{ax} is the allosteric coupling constant, which is defined by (Reinhart, 1983, 1988, 2004; Weber, 1972):

$$Q_{ax} = K_a/K_{a/x} = K_{ix}/K_{ix/a} \quad , \qquad \text{Equation 2}$$

All substitutions at one position were assayed as a group. Each position's group also included a wildtype sample, which gave rise to 9 wildtype replications. These were used to estimate a range for each wildtype parameter (used in below). Wildtype parameters are included in the supplemental material.

Once the 5 functional parameters were determined for each variant at each position, data were analyzed using the RheoScale calculator (Hodges, Fenton, Dougherty, Overholt, & Swint-Kruse, 2018). This program uses all variants available for each position to perform several histogram analyses. The neutral score is the primary focus of the current work. The neutral score is derived from the fraction of variants that fall in the same bin as wildtype protein. To carry out these analyses, the RheoScale calculator has built-in recommendations for bin size and bin number,

but all histogram analyses require empirically assessing the most appropriate bin strategy. Since this was the first study in which we identified positions with strong neutral scores, it was appropriate to further develop the statistical considerations used to generate this score.

In the first iteration of RheoScale (Hodges et al., 2018), experimental error was one of several considerations used to determine bin size. For neutrality, error must be the dominant consideration: All substitutions at a position should have functional parameters that – within error – are equivalent to wildtype protein. To determine this range, we used the nine wildtype replicates and to set the width of the wildtype bin. Data for each position was then analyzed using a "wildtype" bin centered on the wildtype value collected on the same day as that position's variants.

We further considered substitutions with values that fell just outside of the "wildtype" bin. Due to the Gaussian distribution of the range of wildtype values, some variant values that fall just outside of the wildtype bin might be located on the tails of the distribution. Thus, we considered adding "sub-bins" immediately adjacent to each side of the wildtype bin. In contrast to being in the tail of a wildtype distribution, there is some probability that they truly differ from wildtype. Therefore, values in these sub-bins were weighted (with weighting scores less than 1) so that they contributed less to the neutral score. The sub-bins were $1/5^{th}$ the size of the wildtype bin with weights ranging from 0.5 to 0.1. However, these additions had little influence on the final neutral score (supplemental material). Therefore, these modifications were not included in the reported neutral scores.

Results

hLPYK has 543 amino acids. To identify an experimentally tractable number to test for neutrality, we started by considering previous challenges to the assumption that nonconserved positions are neutral. Some nonconserved positions evolve functional variation. For example, some positions show a pattern of amino acid changes that mirrors the overall phylogeny of the protein family (Gu & Vander Velden, 2002; Gu et al., 2013; Lichtarge et al., 1996; Mihalek et al., 2006; Ye et al., 2008). In addition, pairs of nonconserved positions can show correlated changes among their amino acids and that correlation is expected to be associated with some important role for those positions (Brown & Brown, 2010; Dekker et al., 2004; Fodor & Aldrich, 2004; Gobel et al., 1994; Kass & Horovitz, 2002; Olmea et al., 1999; Olmea & Valencia, 1997).

For the PYK family, we performed the analyses that attempt to distinguish nonconserved positions that do and do not have important functions. The outcomes from each perdition were used to generate a composite score that identified the positions *least likely* to exhibit any pattern in its amino acid changes. We followed this by checking all 19 substitutions at each position in a machine learning algorithm – SNAP – that was specifically designed to predict neutral substitutions (Yana Bromberg et al., 2008).

Twenty-two "least-patterned" positions were required to identify 20 hLPYK positions that passed the SNAP test. This list includes: 75, 101, 138, 147, 151, 196, 199, 206, 208, 210, 214, 239, 246, 268, 381, 388, 412, 470, 511, and 517. Before beginning experiments, we next verified that no prior substitutions were documented to alter function by cross-checking against existing experimental data that included: (a) locations of R-PYK missense polymorphisms associated with disease (Pendergrass et al., 2006); (b) positions important to allosteric regulation that were

identified *via* a whole-protein, alanine scan of L-PYK (Tang & Fenton, 2017); and (c) positions in the L-PYK allosteric sites that did not tolerate random mutagenesis (Ishwar et al., 2015; Tang et al., 2017). Most positions passed these criteria, but results from the alanine scan disqualified position 511 (Supplementary data).

From the rest of the list, we noted that positions 196, 199, 206, 208, 210, and 214 were clustered on the primary sequence and we chose to experimentally evaluate 199, 206, 208, 210, and 214. Positions 75, 138, 246, and 412 were selected to represent positions scattered throughout the primary sequence. After experiments were begun, an updated disease database (Canu, De Bonis, Minucci, & Capoluongo, 2016) identified a mutation at position 246 (equivalent to position 277 in the erythrocyte PYK isozyme) (Berghout et al., 2012).

To experimentally test potential neutrality, at least 10 substitutions were introduced at each position (except for 210 which had 9 substitutions; Figure 1). In total, 117 variant proteins were analyzed using >22,000 activity assays to evaluate ligand affinity/binding and allosteric coupling. Quantitative values for each parameter listed in Table 1 were determined for each variant protein (Supplementary information). For each parameter, the data for all variants at each position was then evaluated using the RheoScale calculator, as described in Materials and Methods, to calculate "neutral" scores.

In these calculations, we considered that the definition of neutrality can be either more or a less restrictive. A highly restrictive definition would be requiring a position to be insensitive to all possible substitutions. A less restrictive definition would be to exclude proline and glycine, which alter the polypeptide backbone, from the list of substitutions that can be accommodated at one position. The SNAP predictions also allow for altered function due to tryptophan substitution.

In our study, only 138 and 412 (out of 7 positions for which Pro was obtained as a substitution) retained activity when proline was the replacement. Therefore, data for proline substitutions are included in data tables in the supplement, but were not included in our calculation of neutral scores. In contrast to proline, glycine and tryptophan was accommodated in several positions without altering function. Therefore, glycine and tryptophan substitutions were included in the calculation of neutral scores.

As predicted, substitutions at positions 138, 214, and 246 had little influence on any of the parameters evaluated and therefore, had essentially perfect neutral scores for all parameters (*i.e*, neutral scores of 1) (Figure 2). In addition, we considered that less perfect neutral scores might also reflect a high tendency for neutrality. To that end, we compared the calculated neutral scores (Figure 2) with the primary data (Figure 1). In Figure 2, we also compared outcomes from this study with two previous studies (Hodges et al., 2018; Wu, Swint-Kruse, & Fenton, In preparation) that target sites with known functional importance. Using the RheoScale parameterizations discussed above, the comparison indicates that neutral scores above 0.70 distinguish positions with minimal changes from positions that are known to have functional roles. From this empirical evaluation, any value above 0.70 has a strong neutral score. As such, position 199 has a strong neutral tendency, albeit without perfect neutral scores.

The comparison of the data in Figure 1 with the calculated neutral scores (Figure 2) for $K_{a\text{-}PEP}$ and $Q_{ax\text{-}FBP}$, using position 208 as an example, demonstrates the important role that error/bin size has on calculated neutral scores. The range/bin size for $K_{a\text{-}PEP}$ is small. Therefore, the differences in $K_{a\text{-}PEP}$ among variants at position 208 reduces the neutral score for the evaluation of K_a. Therefore, it is at first surprising that the neutral score for $Q_{ax\text{-}FBP}$ is also not reduced, particularly when recalling the relationship between $K_{a\text{-}PEP}$ and $Q_{ax\text{-}FBP}$ defined in equation 2. In

the evaluation of the neutral score for $Q_{ax\text{-}FBP}$, the larger error for wildtype was largely driven by increased variance in the $K_{a/x\text{-}PEP/FBP}$ value (lower plateau at high Fru-1,6-BP concentration; Figure 1). Nonetheless, independent of the source of the greater error, the resulting greater bin size for $Q_{ax\text{-}FBP}$ accommodates the changes in $K_{a\text{-}PEP}$ and therefore, the neutral score for $Q_{ax\text{-}FBP}$ remains high for position 208.

Discussion

In recent years, dozens of algorithms have been developed to identify "important" amino acid positions and/or to predict harmful amino acid changes (Addington et al., 2013; Adzhubei et al., 2010; Ashkenazy, Erez, Martz, Pupko, & Ben-Tal, 2010; Benítez-Páez, Cárdenas-Brito, & Gutiérrez, 2012; Yana Bromberg et al., 2008; Brown & Brown, 2010; Fodor & Aldrich, 2004; Gray, Kukurba, & Kumar, 2012; Halabi, Rivoire, Leibler, & Ranganathan, 2009; Kc & Livesay, 2011; Mazin et al., 2010; Ng & Henikoff, 2003; Radivojac et al., 2013; Suel, Lockless, Wall, & Ranganathan, 2003; Wilkins, Bachman, Erdin, & Lichtarge, 2012; Ye et al., 2008). However, the reliability of these programs has significant room for improvement (Borras et al., 2012; Dare, Vasta, Penn, Tran, & Hahn, 2013; Domchek & Weber, 2008; Gray et al., 2012; Mauer, Pirzadeh-Miller, Robinson, & Euhus, 2013; Radivojac et al., 2013). One opportunity for improvement arises from exploring the explicit and implicit assumptions upon which algorithms are built. One assumption in many of these analyses is that some protein positions are neutral.

In thinking about this assumption, we traced its history. An early hypothesis about protein evolution was that most amino acid substitutions are neutral, else most of evolutionary changes would be catastrophic(Kimura, 1968). The expanded assumption – that some protein positions are neutral – appears to have derived from protein sequence alignments: Highly conserved positions are thought to reflect the evolutionary pressure of a central role for having a given amino acid at a given position (*e.g.* amino acids with side chains directly involved in active site catalysis). Other evolutionary constraints are hypothesized to be reflected in the patterns of change (co-evolutionary or phylogenetic) observed for some positions. In designating these classes, the (often implicit) corollary has arisen that many nonconserved positions are not important to function.

However, when Bromberg *et al* built a predictor of neutral substitutions, they noted that too few neutral substitutions were documented in the literature to build a gold-standard training set (Y. Bromberg & Rost, 2007; Yana Bromberg et al., 2008; Wainreb et al., 2010). If there is a dearth of documented neutral *substitutions*, there most certainly is a dearth of documented neutral *positions*. Certainly, there are no neutral positions that were proactively predicted to be neutral and then tested. Therefore, the primary goal of this project was to evaluate whether we could experimentally confirm the existence of neutral positions.

This was a daunting proposition. It requires examining multiple variants at multiple protein positions and quantitatively assessing as many functional parameters as possible. The latter is in fact impossible, and in this study, we settled for using a protein for which we could carry out high-throughput mutagenesis on multiple substitutions and perform quantitative assays that report on multiple parameters. It also requires thinking about alternative definitions of neutrality. The definition of neutrality at a single protein level implies that any substitution can be inserted at a position with no change in function. A more relaxed definition for neutrality removes proline and glycine substitutions from consideration due to their ability to modify backbone confirmations. With this the criteria established, we did indeed collect data that are consistent with a few positions being neutral, and the substitution effects on the other positions of this study were lower in magnitude than observed for substitutions at positions near the known allosteric sites (Supplemental Figure).

Another simplification that was required to make the project tractable was that we did NOT generate all possible substitutions at each tested position. Instead, we relied on a random sampling of substitutions. Even so, this study characterized 117 mutant proteins using more than 22,000 assays to evaluate five parameters associated with ligand binding and allosteric functions. Because

it will never be possible to generate all substitutions in all homologs while monitoring all possible functions, we chose to restrict our interpretation: This study has provided evidence that is consistent with the existence of neutral positions. Under these conditions, data for three positions are consistent with their being neutral (positions 138, 214, and 246). This neutrality can be visualized in Figure 2 as neutral scores near one for all five parameters. Position 199 also has high neutral scores, indicating a fourth position of interest. The other positions substituted in this study generally have much less influence on function than the previously substituted positions that are near functional important binding sites (Figure 2) (Wu et al., In preparation).

The risk in using substitution/function sampling to evaluate neutral positions is highlighted by considering the 246 position. Our data is consistent with that position being neutral. However, a more recent addition to the red blood cell pyruvate kinase deficiency data base is equivalent to E246K. (The actual entry is E277K due to the 31 additional N-terminal residues that are absent in hLPYK). Due to the sampling of functions used, it remains possible that an influence of the E246K mutation on k_{cat} is associated with disease. Nonetheless, evaluations of all possible functions will never be possible.

Notably, the bioinformatic analyses used to select the 20 possibly-neutral positions did *not* consider their locations on the protein structure. Therefore, we were surprised at the structural patterns that were apparent when the predicted positions were mapped onto the structure of hLPYK (Holyoak et al., 2013). A large patch of predicted neutral positions (196, 199, 206, 208, 210, 214) resides on the B-domain (Figure 3). The second group of predicted neutral positions showed a pattern when mapped onto the A-domain (Figure 3): The A-domain has a classical TIM barrel fold with eight parallel β-strands that are surrounded by eight α-helices. Interestingly, the TIM barrel in hLPYK is not a continues chain. Instead, the B-domain is inserted the middle of the

sequence that forms the TIM barrel of the A-domain. Despite this separation in sequence space, the predicted neutral positions mapped to the centers of solvent exposed α-helices in the TIM barrel. In a study of other TIM barrel proteins (Chan, Venev, Zeldovich, & Matthews, 2017; Hodges et al., 2018), conserved locations of position types were also observed. It will be interesting to determine whether this is a general pattern for TIM barrels.

Both the interpretation of individual exomes in personalized medicine and efforts to engineer new functions into proteins rely on the ability to accurately predict functional outcomes protein amino acid substitutions. Overall, our data are consistent with the existence of neutral positions. However, as indicated by the E246K example, it will be challenging to assess "all" functions to confirm neutrality. Furthermore, prior to experiments, we put the hLPYK positions through several filters, such that the subset of mutations evaluated would be enriched for neutral positions. Also relevant is that we have not tested all possible functions. Finally, we have made no effort to evaluate if the neutral positions observed in hLPYK can be extrapolated to the equivalent positions in other PYK homologs. Thus, our efforts indicate that neutral positions are rare, at least in hLPYK for which extensive mutational data sets were already available. As such, existing algorithms may over-estimate the presence of functionally neutral positions.

Finally, it is worthwhile to evaluate if the strategy used here to enrich for neutral positions has predictive power that can be used to improve predictive algorithms. The approach we outlined initially identified 20 positions for potential functional neutrality. We can claim some evaluation of 10 of those positions: 1) One was not neutral based on previously observed outcomes from an alanine substitution (Tang & Fenton, 2017). 2) Nine positions were tested here. From this set of 10, we found three to be neutral. However, one of the positions we found to be neutral has now been reported to cause disease, reducing our number to two. The 20% that results from two out of

10 positions being neutral indicates that our approach was not predictive for neutral positions. Nonetheless, by contrasting positions that are known to be important to function (Figure 2), clearly the group of positions selected by our approach is enriched for positions with higher neutral scores).

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Table 1: Parameter Definition	
$K_{a\text{-}PEP}$	PEP apparent affinity
$K_{ix\text{-}FBP}$	Fru-1,6-BP binding
$K_{ix\text{-}Ala}$	Ala binding
$Q_{ax\text{-}FBP}$	Allosteric coupling between PEP binding and Fru-1,6-BP binding
$Q_{ax\text{-}Ala}$	Allosteric coupling between PEP and Ala binding

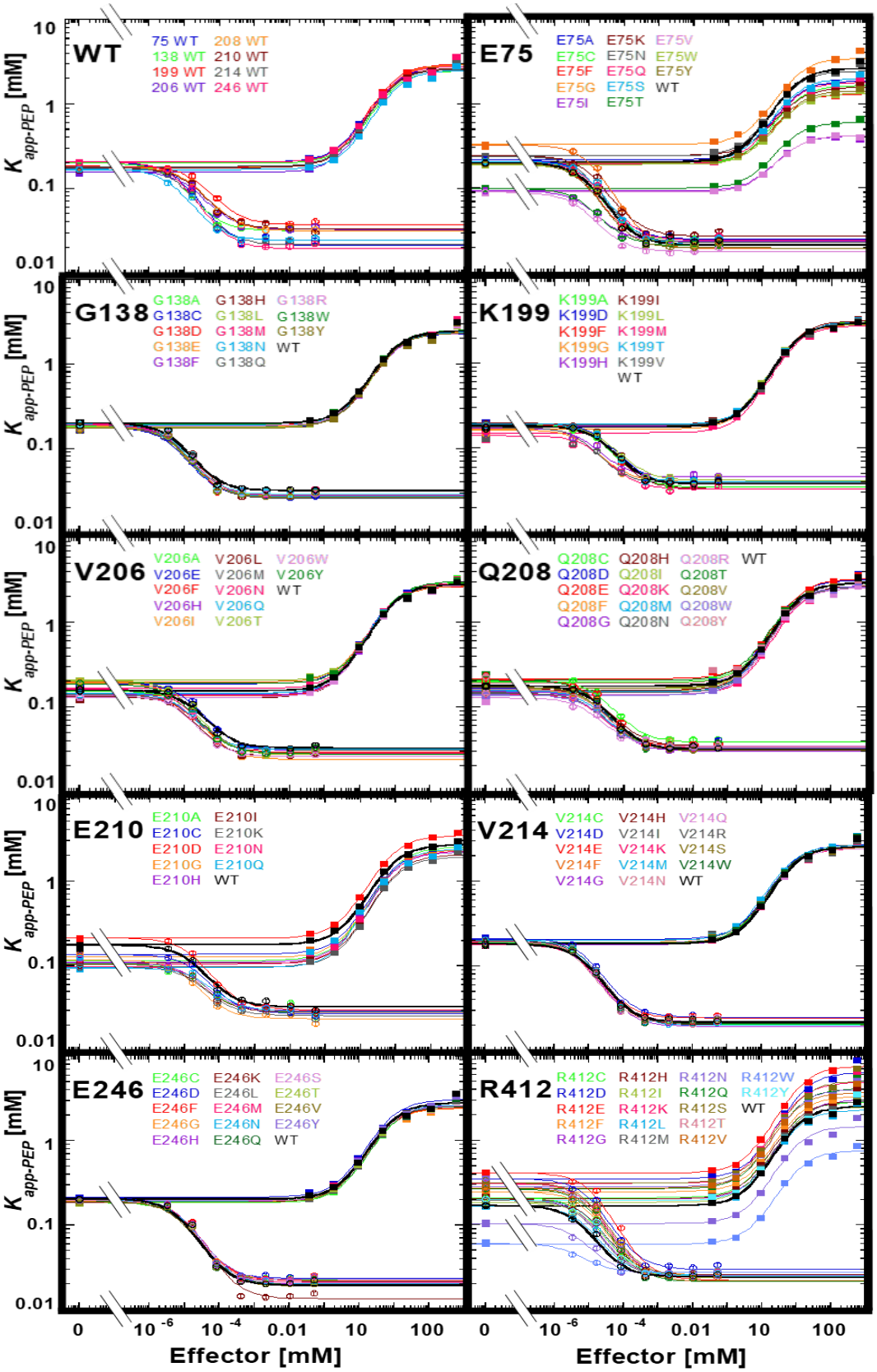
WT
75 WT 208 WT
138 WT 210 WT
199 WT 214 WT
206 WT 246 WT
E75
E75A E75K E75V
E75C E75N E75W
E75F E75Q E75Y
E75G E75S WT
E75I E75T
G138
G138A G138H G138R
G138C G138L G138W
G138D G138M G138Y
G138E G138N WT
G138F G138Q
K199
K199A K199I
K199D K199L
K199F K199M
K199G K199T
K199H K199V
WT
V206
V206A V206L V206W
V206E V206M V206Y
V206F V206N WT
V206H V206Q
V206I V206T
Q208
Q208C Q208H Q208R WT
Q208D Q208I Q208T
Q208E Q208K Q208V
Q208F Q208M Q208W
Q208G Q208N Q208Y
E210
E210A E210I
E210C E210K
E210D E210N
E210G E210Q
E210H WT
V214
V214C V214H V214Q
V214D V214I V214R
V214E V214K V214S
V214F V214M V214W
V214G V214N WT
E246
E246C E246K E246S
E246D E246L E246T
E246F E246M E246V
E246G E246N E246Y
E246H E246Q WT
R412
R412C R412H R412N R412W
R412D R412I R412Q R412Y
R412E R412K R412S WT
R412F R412L R412T
R412G R412M R412V
K app-PEP [mM]
10
1
0.1
0.01
0
10 -6
10 -4
0.01
10
100
Effector [mM]

Figure 1. Functional characterization of hLPYK variants. The response of the apparent affinity of PEP (determined as the concentration of PEP that results in ½ maximal velocity) was measured as a function of effector concentrations for all hLPYK variants. Responses to both the allosteric inhibitor Ala and the allosteric activator Fru-1,6-BP are included. All variants made at one position are included on a single panel. Lines are best fits to equation 1 and all fitted parameters for all variants are listed in Supplemental information. Error bars (most often smaller than the data symbols) are error estimates for the $K_{app\text{-}PEP}$ parameter; when not apparent, error bars are smaller than the symbols. The assay for all variants at a given position were carried out on the same day, along with a wildtype protein that is included in each panel. For comparison of day-to-day variability, all wildtype data sets are shown in the first panel. As described in Materials and Methods, the range of parameters from these wildtype replicates was used to establish bin size in RheoScale calculations.

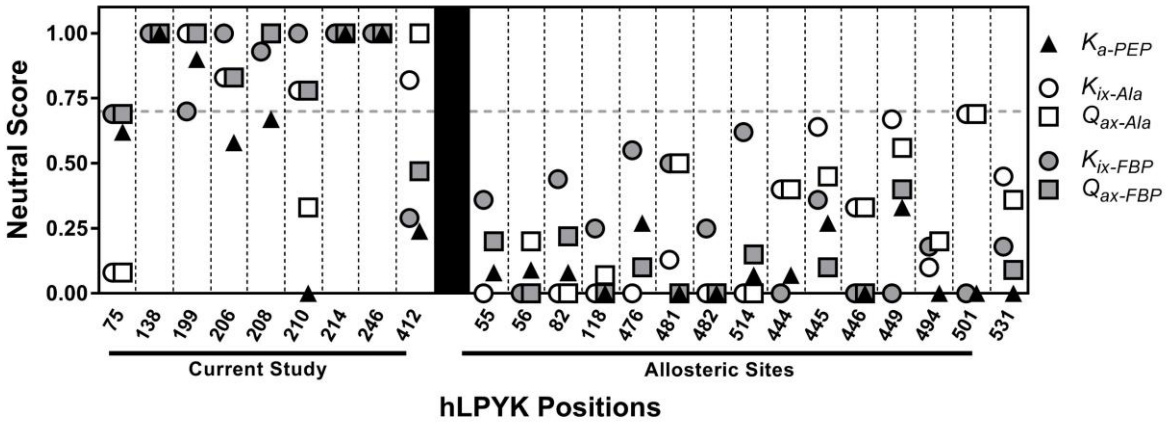

Figure 2. Neutral scores for positions in hLPYK. Left: Parameters derived from data in Figure 1 were used in the RheoScale calculator to quantify the neutral character of each position. The effects of substitutions on PEP affinity, Ala binding ($K_{ix\text{-}Ala}$), PEP/Ala allosteric coupling ($Q_{ax\text{-}Ala}$), Fru-1,6-BP binding ($K_{ix\text{-}FBP}$), and PEP/Fru-1,6-BP allosteric coupling ($Q_{ax\text{-}FBP}$) were used to calculate neutral scores. Positions are noted on the x-axis. Dashed vertical lines separate the scores for individual positions. Right: Neutral scores for positions in allosteric sites evaluated elsewhere (Wu et al., In preparation) and included here for comparison.

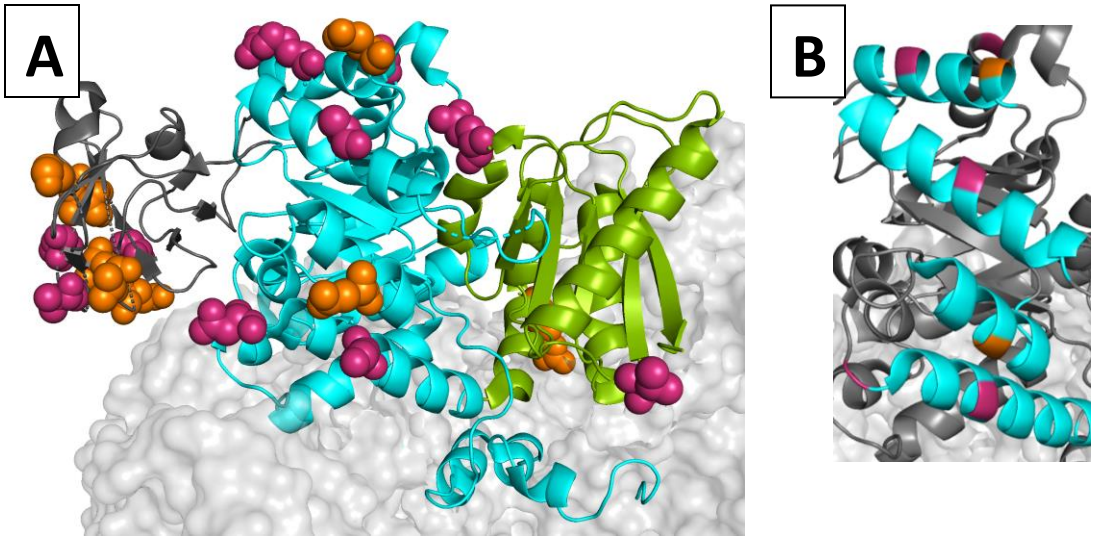

Figure 3. Locations on the hLPYK homotetramer of positions identified as potential neutral. The structure included here is PDB:4IMA (Holyoak et al., 2013). This figures highlights one monomer as a ribbon with neighboring subunits rendered as a light gray surface. A) On the highlighted monomer, its A-, B-, and C- domains are colored cyan, dark gray, and green, respectively. The N-terminal domain is not distinguished from the A domain in this color scheme. The side chains of positions predicted to be neutral positions are in orange and magenta spacefill. Because position 138 is not ordered in this structure, position 137 is highlighted as a proxy. Those positions tested via mutagenesis are in orange. B) Potential neutral positions were patterned in the middle of several helixes from the TIM barrel of the A-domain. TIM barrel helixes are in cyan and identified residues in orange and magenta.