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LACK OF EVIDENCE FOR A ROLE OF CYS-138 AS A BASE CATALYST IN THE SKELETAL MUSCLE 6-PHOSPHOFRUCTO-2-KINASE REACTION

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SUMMARY: The role of Cys-138 in the catalysis of the skeletal muscle 6-phosphofructo-2-kinase reaction was investigated by mutating this residue to serine, glutamine and alanine, expressing the mutants in E. coli with a T7 RNA polymerase-based expression system, and analyzing their kinetic properties. The Cys138Ala mutant had greatly diminished activity, while the Cys138Ser and Cys138Gln mutants had maximal velocities 2-3 fold higher than the wild-type enzyme. It was concluded that Cys-138 does not act as a base catalyst in the kinase reaction, but that it plays a significant structural role in the enzyme's active site.

The bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P_2ase), constitutes an important switching mechanism between glycolysis and gluconeogenesis in liver by catalyzing the formation and degradation of the regulatory metabolite Fru-2,6-P_2 (1). The synthesis and hydrolysis of Fru-2,6-P_2 are catalyzed by a tissue-specific family of at least five mammalian bifunctional isoenzymes including those expressed in liver (1), heart (2-4), brain (5), testes (6), and skeletal muscle (3,7). The rat skeletal muscle bifunctional enzyme is a homodimer with each subunit consisting of a regulatory domain (residues 1-17) and two

Abbreviations used: 6PF-2-K/Fru-2,6-P_2ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; 6PF-1-K, 6-phosphofructo-1-kinase; Fru-2,6-P_2, fructose-2,6-bisphosphate; Fru 6-P, fructose-6-phosphate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PGM, phosphoglycerate mutase; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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independent catalytic domains (8). The bisphosphatase domain (residues 229-448) is structurally similar to phosphoglycerate mutase (9). The kinase domain (residues 18-228) has been predicted by the structural homology model of Bazan *et al.* (9) to form a nucleotide binding fold that is analogous to a segment of both mammalian and bacterial 6PF-1-Ks. This model has been validated with regard to ATP and Fru 6-P binding for 6PF-2-K (8,10). Arg-173 of the skeletal muscle isoform aligns with Arg-162 of 6PF-1-K (9). This is an important 6PF-1-K residue for Fru 6-P binding (11), and has been shown by site-directed mutagenesis to be a crucial Fru 6-P binding residue for 6PF-2-K (8,10). The model of Bazan *et al.* (9) also predicts that Gly-26 of the skeletal muscle isoform would be the first component of a crucial ATP-binding β turn, and this role is supported by site-directed mutagenesis experiments (10). In addition, the model places Cys-138 of skeletal muscle 6PF-2-K in an equivalent position to Asp-127 in *E. coli* 6PF-1-K (9), which has been shown to act as a base catalyst (12). The purpose of these studies was to determine whether Cys-138 acts as a base catalyst in skeletal muscle 6PF-2-K.

MATERIALS AND METHODS

Materials - Restriction enzymes were obtained from New England Biolabs and Boehringer Chemicals. Sephadex G-100 superfine, and Q-sepharose Fast Flow were obtained from Pharmacia LKB Biotechnology Inc. Fru 6-P, Fru-2,6-P_2 and ATP were obtained from Sigma.

DNA Manipulations - Standard DNA manipulations were carried out as described (13). Oligonucleotides were synthesized by phosphoramidite chemistry on an Applied BioSystems Model 381A synthesizer, and purified on oliglnucleotide purification cartridges (OPC) according to the Applied BioSystems manual.

Expression and Purification of Wild-type and Mutant Bifunctional Enzymes in *E. coli* - Expression of mutant and wild-type enzyme forms was accomplished by placing the cDNA for the enzyme of interest into the pET-3a vector, which stands for Protein Expression by T7 RNA Polymerase, developed by Studier and Rosenberg (14). The enzyme extraction method is the same for all the bifunctional enzymes overexpressed in this pET system. Cells were taken at 28 hours after induction with IPTG, which has been shown to be a time at which formation of the soluble skeletal muscle isoenzyme reaches a plateau (8), centrifuged, washed in buffer B (20 mM TES, pH 7.5, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, 2.5 μg leupeptin/ml), pelleted again, and then the pellet from 1 liter of cells was suspended in 30 ml of buffer B containing 0.5 μg/ml lysozyme. The cells were frozen and thawed three times, treated with 0.5 mg of DNAase I in the presence of 5 mM $MgSO_4$ at 4°C for 1-2 hours, and centrifuged (12,000g for 10 minutes), subjected to a 5-20% PEG_{8000}

Table 1

PCR primers for site-directed mutagenesis of skeletal muscle 6PF-2-K/Fru 2,6-P_2ase. The megaprimer was formed in the first PCR reaction with the mutagenic oligonucleotide and the reverse primer using the wild-type cDNA contained in the PET-3a vector as a template. The second PCR reaction of the foward primer and the megaprimer (15) with the same wild-type cDNA template yielded the full length cDNA of the mutants.

Foward	5' TAT AGG GAG ACC ACA A 3'
Reverse	5' GGC GTA TCA CGA GGC C 3'
WT	5' --- --- --- TGT --- --- 3'
Cys138Ala	5' GAG TCT ATT GCT AAT GAC C 3'
Cys138Ser	5' GAG TCT ATT AGT AAT GAC C 3'
Cys138Gln	5' GAG TCT ATT CAG AAT GAC C 3'

fractionation, and then purified using a 100-500 mM KCl gradient at pH 7.4 on a Q-sepharose column, assayed for soluble 6PF-2-K/Fru-2,6-P_2ase by the E-P assay (2), pooled and then further purified on a G100 gel filtration column, as described previously (8).

Site-Directed Mutagenesis - PCR (15) oligonucleotide-directed megaprimer mutagenesis, which employed the pET-3a expression vector encoding the wild-type bifunctional enzyme (pPKBET), was used for mutant construction, as previously described (8). This PCR mutagenesis method used a single stranded mutant primer and an amplifying primer which each anneal to a single strand of the duplex wild-type cDNA during the PCR cycle, and are then extended by the action of thermostable Taq polymerase. Table 1 shows the 5' and 3' PCR primer for the oligonucleotide-directed PCR mutagenesis reactions, as well as the 5' primer for the following megaprimer PCR reaction. The PCR reactions used a denaturation temperature of 94°C for 30 seconds, an annealing temperature of 55°C, for 30 seconds, and an extension temperature of 72°C for 30 seconds over 25 PCR cycles. The product of the oligonucleotide-directed PCR reaction was then ethanol precipitated, dissolved in TE buffer, and then extended in the megaprimer PCR reaction, which results in a final cDNA product spanning the entire kinase domain. The product of the megaprimer PCR reaction was then ethanol precipitated, digested with Xho I and Nco I to form a "cassette" of the kinase region, and this "cassette" was then ligated into pPKBET. All mutations were confirmed by DNA sequencing by the method of Sanger and Coulson (16).

Assay of Enzyme Activity - 6PF-2-K activity was measured at pH 7.5 by monitoring the production of Fru-2,6-P_2 in buffer C (100 mM Bis Tris Propane/Na acetate 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, 2.5 µg/ml leupeptin and 5 mM potassium phosphate). Phosphoenzyme formation was assayed as acid-precipitable radioactivity on phosphocellulose paper (2).

Kinetic Analysis - Calculations of V_{max} and K_m for the enzymes described herein, as well as the generation of 6PF-2-K and Fru-2,6-

P_2ase kinetic graphs, were made using the linear and nonlinear regression subroutines of the GraphPAD Inplot program (18).

Other Methods - [2-^{32}]Fru-2,6-P_2 was prepared enzymatically from [γ-^{32}P]ATP and Fru 6-P as described previously (19). Protein was determined with BCA protein assay reagent from Pierce, Inc., Rockford, IL.

RESULTS

Effect of Cys-138 Mutations on 6PF-2-K Protein Expression - The skeletal muscle isoform and its mutants were expressed and purified as previously described (8), and the amount of active, soluble bifunctional enzyme produced was 10-15 mg/liter for the wild-type, Cys138Ser and Cys138Gln mutants. However, active soluble Cys138Ala was produced at levels of approximately 1 mg/liter. This 10-fold decrease in the amount of active Cys160Ala produced, in comparison to the wild-type enzyme, is consistent with a structural role for Cys-138 in the maintenance of 6PF-2-K stability.

Effect of Cys-138 Mutations on 6PF-2-K Substrate Dependence and Activity - Four sulfhydryl residues (including Cys-138), out of the twelve present in the bifunctional enzyme, are essential for catalysis of the kinase reaction (20). Oxidation of 6PF-2-K/Fru-2,6-P_2ase with a variety of reagents, such as mixed function oxidation systems (e.g. Fe + ascorbate + perchlorate) resulted in the complete loss of kinase activity but had no effect on bisphosphatase activity (21,22). Dithiothreitol completely prevented the inactivation of 6PF-2-K when added before ascorbate/Fe^{+++} and restored activity when added after its inactivation, indicating that these cysteinyl residues are essential for Fru 6-P binding and/or transfer of the γ-phosphate of ATP to the 2-hydroxyl of Fru 6-P (21). Neither Fru 6-P nor Fru-2,6-P_2 afforded any protection against the oxidation (21), raising the issue of whether these cysteinyl residues are directly involved in sugar phosphate binding and/or play a structural role. ATP prevented the oxidation-induced inactivation as well as subsequent inactivation when added to the partially oxidized enzyme, suggesting that one or more of these cysteinyl residues is near the active site (21). Carboxyamidomethylation of these cysteinyl residues by iodoacetamide enhances the turnover number (k_{cat}) by 20-fold thru an unknown mechanism which may involve structural changes within the active site (22).

Table 2

Comparison of the 6-PF-2-K activities of the skeletal muscle wild-type and its Cys-138Ser and Cys138Gln mutants. V_{max} and K_m values were obtained in the presence of 5 mM potassium phosphate using the nonlinear regression routinues of GraphPAD InPlot (18) from the data displayed in Figures 1 and 2. 6PF-2-K activity was assayed as described in **Methods**.

Enzyme form	V_{max} (mU/mg)	K_m(Fru 6-P) (μM)	K_m (ATP) (μM)	n_h ATP
Wild-type	11.1 ± 1.7	570 ± 100	290 ± 60	1.14
Cys138Ser	30.6 ± 1.8	1100 ± 150	490 ± 50	1.28
Cys138Gln	28.2 ± 2.3	490 ± 100	1100 ± 100	1.08
Cys138Ala	~0.1	-	-	-

Since the model of Bazan *et al.* (9) had placed Cys-138 in the skeletal muscle enzyme in an equivalent position to Asp-127, a base catalyst in bacterial 6PF-1-K, Cys-138 was mutated to alanine, serine and glutamine. If Cys-138 functions primarily as a base catalyst, mutation to alanine or serine should result in a 10^4-10^5-fold decrease in activity. If Cys-138 has a role in Fru 6-P binding, its mutation to serine would not be expected to change the enzymes' affinity for Fru 6-P, since a cysteine to serine mutation is a conservative change. However, if Cys-138 has an important structural role, mutation of this residue would be expected to affect 6PF-2-K function. In addition, the long side chain of the glutamine residue in Cys138Gln may mimic carboxyamidomethylation of this cysteinyl residue by iodoacetamide and cause an increase in activity.

As shown in Table 2, the mutation of Cys-138 to alanine resulted in a 100-fold decrease in 6PF-2-K activity, while mutation of Cys-138 to serine or glutamine resulted in a 3-fold increase in activity. The K_m for Fru 6-P is identical for the Cys138Gln and wild-type enzymes, but was increased 2-fold for the Cys138Ser mutant in comparison to the wild-type (Figure 1). In comparison to the wild-type, the K_m for ATP was increased roughly 2-fold for the Cys138Ser mutant and 4-fold for the Cys138Gln mutant (Figure 2). The substrate dependence of the 6PF-2-K velocity on Fru 6-P or ATP was hyperbolic for the wild-type and all mutant enzymes (Table 2).

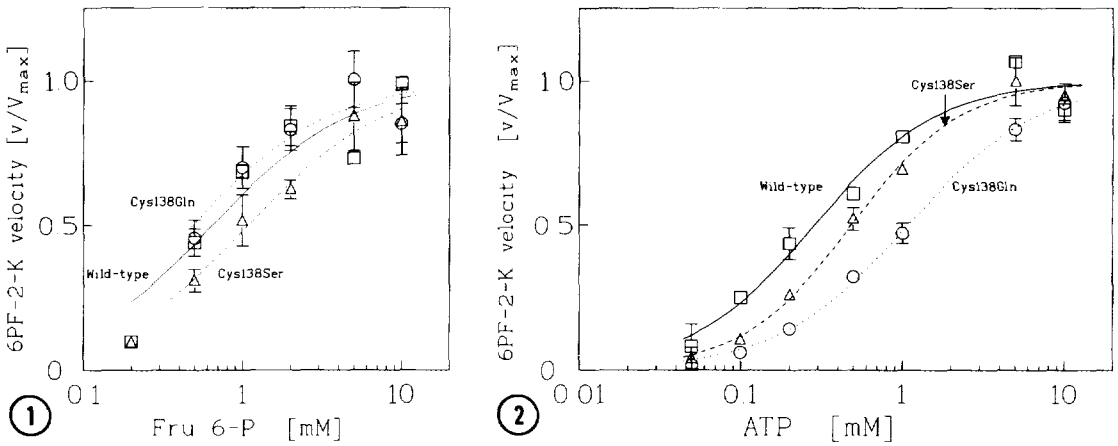

Figure 1. The Fru 6-P dependence of the recombinant skeletal muscle isoform and its Cys138Ser and Cys138Gln mutants. 6PF-2-K activity was determined at pH 7.5 in the presence of 5 mM potassium phosphate and 5 mM ATP, at the indicated concentration of Fru 6-P, as described under "Materials and Methods". Measurements are the average of at least three determinations ± standard error of the mean. □ Skeletal muscle wild-type, ○, Cys138Gln, and △ Cys138Ser.

Figure 2. The ATP dependence of the recombinant skeletal muscle isoform, and its Cys138Ser and Cys138Gln mutants. 6PF-2-K activity was determined in the presence of 5 mM potassium phosphate and 10 mM Fru 6-P at the indicated concentration of ATP, as described under "Materials and Methods". Measurements are the average of at least three determinations ± standard error of the mean. □, Skeletal muscle wild-type, ○, Cys138Gln, and △, Cys138Ser.

DISCUSSION

Although mutation of Cys-138 to alanine resulted in a mutant with no significant 6PF-2-K activity, mutation of this residue to serine resulted in a 3-fold increase in its maximal velocity, indicating that this residue is not functioning as a base catalyst. A similar mutation of the base catalyst in bacterial 6PF-1-K, Asp127Ser, resulted in a 10,000-fold decrease in its maximal velocity (12). The specific activity of mammalian 6PF-2-K is very low, with liver 6PF-2-K having a k_{cat} (0.1 sec -1) three orders of magnitude less than that of mammalian 6PF-1-K (1). Interestingly, the maximal velocity of the bacterial Asp127Ser 6PF-1-K mutant is roughly equivalent to that of the wild-type skeletal muscle 6PF-2-K, which suggests that the 6PF-2-K reaction may proceed without the intervention of a strong base catalyst. In that case, catalysis of the 6PF-2-K reaction may be mediated purely by preferential binding of the substrates in the transition state compared to the ground

state, similar to the mechanism of stabilization of enzyme transition states by catalytic antibodies (23,24).

The maximal velocity of the liver 6PF-2-K is approximately 2-fold higher than that of the skeletal muscle 6PF-2-K (8). When the corresponding cysteinyl residue (Cys-160) was mutated to aspartate in the liver isoform, a 10-fold decrease in 6PF-2-K activity was observed, rather than an increase in activity which would be expected if this residue functioned as a base catalyst (unpublished results). The geometry of the active site region of the skeletal muscle and liver enzymes may be different, as evidenced by the latter enzyme's higher V_{max} and lower K_m for Fru 6-P (8). Furthermore, mutation of this cysteinyl residue to alanine in the skeletal muscle enzyme resulted in a large decrease in maximal velocity, as well as in the amount of active enzyme formed, suggesting its importance as a structural residue. In support of a critical structural role for this cysteinyl residue, alignment of the liver, heart, brain, testes and yeast 6PF-2-K/Fru 2,6-P_2ases indicates that of all the cysteinyl residues shown to be important for kinase activity in the mammalian 6PF-2-Ks, only this residue is conserved in the yeast 6PF-2-K (25). The observation that the Cys138Gln mutant has a 4-fold increase in its K_m for ATP suggests that the residue may lie near an important ATP binding residue in the active site, as indicated by previous chemical modification studies (21).

Experiments in which 6PF-2-K was modified by various reagents implicate cysteinyl, and possibly several other residues as being important for 6PF-2-K activity (20-22). The active site of bacterial 6PF-1-K has several residues which play a catalytic role in addition to the strong base catalyst Asp-127 (26). The model of Bazan *et al.* (9) predicts that the active site of 6PF-2-K includes the region around Cys-138, but that Asp-140, Asp-108 and Arg-82 may also be important in catalysis. The results of this study support the notion that the active site includes Cys-138 in skeletal muscle (and by extension Cys-160 in the liver enzyme), but indicates that if there is a base catalyst and/or other catalytic residues in this reaction, they do not include this cysteinyl residue. Studies to identify catalytic residues in both the skeletal muscle and liver isoforms are in progress.

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