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Lysine-21 of *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase participates in substrate binding through charge–charge interaction

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Abstract

Leuconostoc mesenteroides glucose 6-phosphate dehydrogenase (G6PD) was isolated in high yield and purified to homogeneity from a newly constructed strain of *Escherichia coli* which lacks its own glucose 6-phosphate dehydrogenase gene. Lys-21 is one of two lysyl residues in the enzyme previously modified by the affinity labels pyridoxal 5′-phosphate and pyridoxal 5′-diphosphate-5′-adenosine, which are competitive inhibitors of the enzyme with respect to glucose 6-phosphate (LaDine, J.R., Carlow, D., Lee, W.T., Cross, R.L., Flynn, T.G., & Levy, H.R., 1991, *J. Biol. Chem. 266*, 5558–5562). K21R and K21Q mutants of the enzyme were purified to homogeneity and characterized kinetically to determine the function of Lys-21. Both mutant enzymes showed increased K_m-values for glucose 6-phosphate compared to wild-type enzyme: 1.4-fold (NAD-linked reaction) and 2.1-fold (NADP-linked reaction) for the K21R enzyme, and 36-fold (NAD-linked reaction) and 53-fold (NADP-linked reaction) for the K21Q enzyme. The K_m for $NADP^+$ was unchanged in both mutant enzymes. The K_m for NAD^+ was increased 1.5- and 3.2-fold, compared to the wild-type enzyme, in the K21R and K21Q enzymes, respectively. For the K21R enzyme the k_{cat} for the NAD- and NADP-linked reactions was unchanged. The k_{cat} for the K21Q enzyme was increased in the NAD-linked reaction by 26% and decreased by 30% in the NADP-linked reaction from the values for the wild-type enzyme. The data are consistent with Lys-21 participating in the binding of the phosphate group of the substrate to the enzyme via charge–charge interaction.

Keywords: active-site glucose 6-phosphate dehydrogenase; glucose 6-phosphate dehydrogenase; kinetic analysis; *Leuconostoc mesenteroides*; lysine; site-directed mutagenesis; substrate binding

Glucose 6-phosphate dehydrogenase (G6PD) from *Leuconostoc mesenteroides* can utilize either NAD^+ or $NADP^+$ in the oxidation of glucose 6-phosphate (G6P) (DeMoss et al., 1953). The dual coenzyme specificity is unusual among dehydrogenases, most of which strongly prefer either NAD^+ or $NADP^+$. The enzyme is a dimer composed of identical subunits of molecular weight 54,316 (Lee et al., 1991). The X-ray crystal structure of the enzyme is currently under investigation (Adams et al., 1983).

The kinetic mechanisms for the enzyme differ depending on the coenzyme that is used. In the NAD-linked reaction either NAD^+ or G6P can bind first in a random sequential mechanism, and NAD^+ and G6P promote each other's binding. In the NADP-linked reaction there is no mutual interaction between the binding of $NADP^+$ and G6P, and the kinetic mechanism is ordered sequentially with coenzyme binding first (Olive et al., 1971; Levy et al., 1983). These different kinetic mechanisms may be important in the regulation of the enzyme, as suggested by dual wavelength assays using thionicotinamide analogs of the coenzymes (Levy & Daouk, 1979; Levy et al., 1979).

Both pyridoxal 5′-phosphate (PLP) and pyridoxal 5′-diphospho-5′-adenosine (PLP-AMP) inhibit the enzyme competitively with respect to G6P and noncompetitively with respect to NAD^+ and $NADP^+$ (LaDine et al., 1991). Studies with the ^3H-labeled affinity analogs showed that two lysyl residues, Lys-21 and Lys-343, were modified following reduction with $NaBH_4$ (LaDine et al., 1991). G6P protects the enzyme against covalent modification of both lysyl residues by PLP and PLP-AMP.

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Furthermore, NAD^+ protects the enzyme as effectively as G6P against such covalent modification, presumably due to the well-documented conformational change engendered by NAD^+ binding to *L. mesenteroides* G6PD (Haghighi & Levy, 1982; Kurlandsky et al., 1988).

We have previously reported the cloning of the *L. mesenteroides* G6PD gene and its expression in *Escherichia coli* (Lee et al., 1991). We report here the purification of *L. mesenteroides* G6PD from a strain of *E. coli* that contains a deletion in its G6PD gene, using the dye-ligand chromatography method of Hey and Dean (1983). We have also mutated Lys-21 of G6PD, to study the effects of the positive charge of its ϵ-amino group, to Arg, Gln, and Glu. These mutant enzymes were purified and characterized kinetically and the results presented here indicate that Lys-21 is involved in G6P binding, probably through charge–charge interaction.

Results and discussion

Isolation of L. mesenteroides G6PD from E. coli

In order to successfully perform site-directed mutagenesis experiments on the *L. mesenteroides* G6PD for kinetic and crystallographic studies it is necessary to isolate large amounts of the enzyme from *E. coli*. The gene encoding *L. mesenteroides* G6PD was cloned into pUC19 and, fortunately, the resulting recombinant plasmid, pLmz, expresses the enzyme in *E. coli* (Lee et al., 1991), making an expression plasmid unnecessary. *Escherichia coli* strain SU294 contains a deletion in its G6PD gene and, when transformed with pLmz, is an excellent source of *L. mesenteroides* G6PD. Approximately 15% of the protein in the crude extract from the transformed cells is *L. mesenteroides* G6PD, and the enzyme is readily purified using a two-step ammonium sulfate fractionation followed by the two dye-ligand column purification scheme of Hey and Dean (1983). The *L. mesenteroides* G6PD eluted from the Matrex Gel Orange B column by a pulse of $NADP^+$ is judged to be homogeneous as seen by SDS-PAGE (Fig. 1). Starting with a 250-mL culture of cells, 2–3 mg of enzyme are routinely purified. Table 1 shows the yield of the *L. mesenteroides* G6PD at each step of the purification. The kinetic values obtained for the wild-type enzyme isolated from *E. coli* (Tables 2, 3) are 1.3–1.5-fold higher than those previously published for the enzyme isolated from *L. mesenteroides* (Olive et al., 1971). The specific activity of the recombinant enzyme is also significantly higher than the values obtained from commercial enzyme, which may be due to heterogeneity present in the latter (see Lee et al., 1991).

Table 1. *Summary of Leuconostoc mesenteroides G6PD purification from Escherichia coli*

	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	3,660	35.7	102	100
50–100% $(NH_4)_2SO_4$	2,320	14.4	161	63.4
Matrex Gel Purple A	1,770	3.20	554	48.4
Matrex Gel Orange B	1,680	2.35	715	45.9

[a] One unit is the amount of enzyme that catalyzes the formation of 1 μmol NADPH/min under standard assay conditions (see Materials and methods).

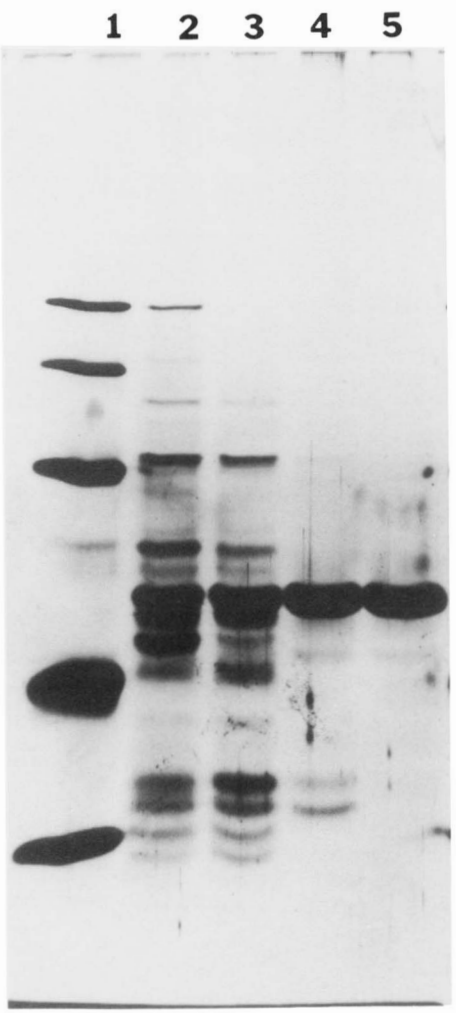

Fig. 1. Purification of *Leuconostoc mesenteroides* G6PD from *Escherichia coli*. SDS-PAGE after each step in the purification of *L. mesenteroides* G6PD from *E. coli* strain SU294 containing pLmz. Lane 1 contains standard proteins (molecular weights in parentheses): carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase b (97,400), and β-galactosidase (116,000); lane 2: crude extract; lane 3: 50–100% ammonium sulfate-precipitated protein; lane 4: protein passing through the Matrex Gel Purple A column; and lane 5: enzyme eluted from Matrex Gel Orange B column. A sample of 1.0 μg of protein was loaded per lane and the gel was silver stained (Merril, 1990).

Table 2. *Kinetic data for the NAD-linked reactions for the Leuconostoc mesenteroides wild-type enzyme from Escherichia coli and the K21R and K21Q mutant G6PDs*[a]

Enzyme	K_m G6P (μM)	K_m NAD^+ (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (G6P) (min^{-1} μM^{-1})[b]
Wild type	69.2 (9.24)	162 (18.1)	67,500	975
K21R	96.5 (11.1)	240 (25.8)	66,100	685
K21Q	2,500 (200)	518 (72.4)	85,400	34.2

[a] The standard errors are given in parentheses.
[b] Per mole of enzyme subunit.

Isolation and kinetic analysis of the K21R, K21Q, and K21E G6PDs

The K21R, K21Q, and K21E enzymes were isolated from *E. coli* strain SU294 using the same protocol described above for the wild-type enzyme. After the wild-type, K21R, and K21Q enzymes were purified to homogeneity they were characterized kinetically to determine their K_m and k_{cat} values. The K21E enzyme was purified, but had very little enzyme activity (0.13% of the NADP-linked activity compared to the wild-type enzyme under standard assay conditions) and was not analyzed kinetically. The kinetic data for the NAD-linked reactions (Table 2) show that the K_m for G6P is increased 1.4- and 36-fold for the K21R and K21Q enzymes, respectively, compared to the K_m for G6P in the wild-type enzyme. The K_m for NAD^+ is increased 1.5-fold for the K21R enzyme and 3.2-fold for the K21Q enzyme. The k_{cat} was unchanged for the K21R enzyme, but for the K21Q enzyme the k_{cat} is increased by 26% compared to the k_{cat} of the wild-type enzyme. In the NADP-linked reaction (Table 3) the K21R enzyme has a 2.1-fold increase in the K_m for G6P, while the K21Q enzyme has a 53-fold increase in the K_m for G6P, compared to the value for wild-type enzyme. The K_m and K_i values for $NADP^+$ are essentially unchanged in both mutant enzymes. The k_{cat} value is unchanged in the K21R enzyme, but is decreased by 30% compared to the wild-type enzyme for the K21Q mutant.

Kinetic and binding studies have shown that NAD^+ and G6P promote each other's binding to *L. mesenteroides* G6PD, whereas $NADP^+$ and G6P do not influence each other's binding to the enzyme (Olive et al., 1971). This accounts for the fact that the K_m for NAD^+ is increased slightly in the K21R and K21Q G6PDs. It also explains why the K_m values for G6P in these enzymes are less affected in the NAD-linked than in the NADP-linked reaction.

Lys-21 was mutated to arginine, glutamine, and glutamate to study the effect of the positive charge of the ϵ-amino group on the binding of G6P. The K21R enzyme, which retains the positive charge on its side chain, is most similar to the wild-type enzyme: its k_{cat} is unchanged while the K_m for G6P is increased slightly in both the NAD- and NADP-linked reactions. The K_m for NAD^+ is also increased slightly while the K_m for $NADP^+$ is unchanged. The K21Q enzyme is much less efficient than the wild-type enzyme: the K_m for G6P is greatly increased in both the NAD- and NADP-linked reactions. The K_m for NAD^+ is increased slightly, whereas it is unchanged for $NADP^+$. The K21E enzyme has very little activity in both the NAD- and NADP-linked reactions. These data indicate that Lys-21 interacts with G6P, probably through charge–charge interaction. This interpretation is also consistent with kinetic studies performed on the enzyme at different pH values. Olive et al. (1971) found that a group on the enzyme with a pK′ of 8.9 was involved in binding G6P. The K21R enzyme shows only a small increase in the K_m for G6P, whereas it is greatly increased for the K21Q mutant, indicating a decrease in the ability of the enzyme to bind G6P, presumably due to the loss of the positive charge on the side chain in K21Q. The low activity of the K21E enzyme is, therefore, not surprising as charge–charge repulsion between the enzyme and G6P may be occurring in this enzyme.

From these data one can calculate the contribution that Lys-21 makes to the binding of G6P in the transition state. Assuming that the side chain of Lys-21 is involved in binding, and not in catalysis, it can be shown that:

$$\Delta G_R = RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$$

Table 3. *Kinetic data for the NADP-linked reaction of the Leuconostoc mesenteroides wild-type enzyme from Escherichia coli and for the K21R and K21Q mutant G6PDs*[a]

Enzyme	K_m G6P (μM)	K_m $NADP^+$ (μM)	K_i ($NADP^+$) (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (G6P) (min^{-1} μM^{-1})[b]
Wild type	114 (10.5)	7.99 (0.722)	3.37 (1.23)	31,300	275
K21R	239 (17.6)	8.08 (0.529)	6.20 (1.18)	34,900	146
K21Q	6,070 (575)	7.31 (1.07)	7.77 (1.55)	21,900	3.61

[a] The standard errors are given in parentheses.
[b] Per mole of enzyme subunit.

(Wilkinson et al., 1983), where ΔG_R is the binding energy of the Lys-21 side chain in the transition state, and mut and wt refer to the mutant and wild-type enzymes, respectively. Applying this equation to the binding of G6P in the wild-type enzyme and the K21Q mutant, $\Delta G_R = -2.57$ kcal/mol in the NADP-linked reaction at 25 °C. Implicit in this calculation are the assumptions (Wilkinson et al., 1983) that the side chain of Gln-21 does not participate in G6P binding and that the mutation affects the binding of only G6P, not coenzyme, an assumption justified for the NADP-linked reaction because the K_m for $NADP^+$ is unaffected by the mutation (Table 3). The above equation is not applicable to the NAD-linked reaction because the K_m's for both NAD^+ and G6P are affected by the mutation. The contribution of 2.57 kcal/mol to G6P binding by the side chain of Lys-21 may be compared to the value of 2.9 kcal/mol for the salt bridge between the α-NH_3^+ group of Ile-16 and the CO_2^- group of Asp-194 in the catalytically active conformation of chymotrypsin (Fersht, 1972).

Lys-21 is conserved in all known G6PD sequences (Fig. 2) except that of the *Zymomonas mobilis* enzyme, which is also dual coenzyme-specific. It is noteworthy (see Fig. 2) that in all G6PDs, except that from *Z. mobilis*, this region contains a conserved sequence, GlyXxxXxx GlyXxxXxxAla$(Xxx)_{12}$Lys/Arg, which is a slight modification of the consensus NADP-binding sequence, GlyXxxGlyXxxXxxAla$(Xxx)_{12}$Lys/Arg, seen in several NADP-linked dehydrogenases (Hanukoglu & Gutfinger, 1989). When solved, the X-ray structure of *L. mesenteroides* G6PD should reveal whether this region of the enzyme participates in binding both substrate and coenzyme.

Materials and methods

Materials

Restriction enzymes were from Bethesda Research Laboratories, modified T_4 DNA polymerase (Sequenase) from U.S. Biochemical, [^{35}S]-dATP for DNA sequencing from New England Nuclear, Coomassie Protein Assay Reagent from Pierce, and the oligonucleotide-directed in vitro mutagenesis system version 2 from Amersham. NAD^+ and $NADP^+$ were from Boehringer Mannheim, and G6P and hen egg white lysozyme were from Sigma. The oligonucleotides for the K21R mutation (5′ACTTG GCCAAGCGTCGTCTTTACCCATCAGT 3′) and the K21E/Q mutation (5′ GCCAAGCGTC/GAGCTTTAC 3′) were synthesized at the Syracuse University DNA and Protein Core Facility. Twice-distilled water was used in all experiments. All other reagents were analytical grade.

H.	(39)	G	A	S	G	D	L	A	K	K	K	I	Y	P	T	I	W	W	L	F	R
R.	(39)	G	A	S	G	D	L	A	K	K	K	I	Y	P	T	I	W	W	L	F	R
D.	(43)	G	A	S	G	D	L	A	K	K	K	I	Y	P	T	L	W	W	L	Y	R
S.	(18)	G	A	S	G	D	L	A	K	K	K	T	F	P	A	L	F	G	L	F	R
E.	(16)	G	A	K	G	D	L	A	R	R	K	L	L	P	S	L	Y	Q	L	E	K
Z.	(12)	G	S	T	G	D	L	S	Q	R	M	L	L	P	S	L	Y	G	L	D	A
L.	(12)	G	G	T	G	D	L	A	K	R	K	L	Y	P	S	V	F	N	L	Y	K

Fig. 2. Amino acid sequence around Lys-21 in *Leuconostoc mesenteroides* G6PD and homologous sequences in other G6PDs. The sequences are from human (H) (Persico et al., 1986), rat (R) (Ho et al., 1988), *Drosophila melanogaster* (D) (Fouts et al., 1988), *Saccharomyces cerevisiae* (S) (Nogae & Johnston, 1990; Thomas et al., 1991), *Escherichia coli* (E) (Rowley & Wolf, 1991), *Zymomonas mobilis* (Z) (Barnell et al., 1990), and *L. mesenteroides* (L) (Lee et al., 1991). The numbers in parentheses indicate the number of the first amino acid in each sequence. Lys-21 of the *L. mesenteroides* G6PD is boxed.

Bacterial strains

Strain SU294 was constructed in the following manner. A P1v transducing lysate was prepared on *E. coli* strain DC 374 (*zea*-2222::Tn*10*, *fadD*, *mel-1*, *supF*58), a strain which has transposon Tn*10* linked to *zwf*, the structural gene for G6PD. This lysate was used to transduce strain DF1671 DZ1 (*hisG*1, Δ[*eda-zwf*]15, *rpsL*115, *pgi*-2), a variant in which the *zwf* gene is deleted, but which could not be used to isolate *L. mesenteroides* G6PD because pLmz could not be maintained in it. Transductants were selected for resistance to tetracycline. A second P1v lysate was made on one of the tetracycline-resistant transductants which had retained the *eda–zwf* deletion. This lysate was used to transduce *E. coli* strain MM294 (*supE*44, *hsdR*, *endA*1, *pro*, *thi*), again selecting for tetracycline resistance. Transductants were individually screened to determine which ones did not contain G6PD activity, and thus had been cotransduced for Tn*10* and the *eda–zwf* deletion. Strain SU294 was one of these and has the presumptive genotype *zea*-2222::Tn*10*, *supE*44, *hsdR*, *endA*1, *pro*, *thi*, Δ(*eda–zwf*)15.

DNA techniques

Plasmid transformations (Hanahan, 1985) were performed as described. The procedures of Sambrook et al. (1989) were used for standard DNA protocols. DNA sequencing was done on single-stranded M13 subclones using Sequenase.

Site-directed mutagenesis

The K21R, K21Q, and K21E mutants were constructed with the oligonucleotides listed above using the Amersham oligonucleotide-directed in vitro mutagenesis system version 2. After isolation of the mutants, each one was sequenced to confirm the presence of the desired mutation and the absence of any other mutations in the gene. The K21R, K21Q, and K21E enzymes were isolated, after subcloning the mutant genes into pUC19, from *E. coli* strain SU294 by applying the same protocol used to isolate the wild-type enzyme (see below).

Isolation and purification of L. mesenteroides G6PD from E. coli

A 250-mL culture of *E. coli* strain SU294 containing pLmz was grown overnight at 37 °C with shaking. Cells were harvested by centrifugation at 6,000 rpm in a GSA rotor in a refrigerated RC-5B ultracentrifuge for 10 min. The cells were washed with 25 mL of a solution containing 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH 8.0, and recentrifuged at the same speed in an SS34 rotor. The cells were resuspended in 5 mL of a solution containing 8% sucrose, 0.1% Triton X-100, 50 mM EDTA, pH 8.0, and 10 mM Tris-HCl, pH 8.0, to which 100 μL of a fresh lysozyme solution (100 mg/mL in 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, pH 8.0) was added. After a 30-min incubation the lysed cells were centrifuged at 18,000 rpm in an SS34 rotor for 20 min. The supernatant was saved and the cellular debris was resuspended in 80 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8.[1] After a 30-min incubation the cellular debris was centrifuged at 18,000 rpm in an SS34 rotor for 20 min, and the supernatant was combined with the supernatant from the previous spin. The supernatants, which contain the enzyme activity, were dialyzed against 50 mM Tris-HCl, pH 7.8. After dialysis the crude extract was centrifuged to remove insoluble material. Ammonium sulfate (enzyme grade) was then added to 50% saturation at 0 °C and the precipitated protein was removed by centrifugation. The ammonium sulfate concentration was brought to saturation at 0 °C and the precipitated protein, which contained the G6PD activity, was redissolved in 50 mM Tris-HCl, pH 7.8, and dialyzed against the same buffer. The enzyme was further purified by the two dye-ligand column purification procedure of Hey and Dean (1983), using 45 mL each of Matrex Gel Purple A and Matrex Gel Orange B. G6PD eluted from the Matrex Gel Orange B column is homogeneous as seen by SDS-PAGE (Fig. 1). Protein concentration was determined with the Coomassie Protein Assay Reagent using bovine serum albumin as a standard.

Assays of G6PD activity

Enzyme activity was routinely measured at 25 °C by following the appearance of NAD(P)H at 340 nm. Assays were initiated by the addition of enzyme to 1.0 mL of solution containing 33 mM Tris-HCl (pH 7.6), 1.6 mM glucose 6-phosphate, and 114 μM $NADP^+$, or for the NAD-linked reaction, 33 mM Tris-HCl (pH 7.6), 1.06 mM G6P, and 21.2 mM NAD^+ (neutralized to pH 7.0).

Kinetic analysis of enzymes

The recombinant enzyme from *E. coli* and the site-directed mutant enzymes K21R and K21Q were subjected to kinetic measurements performed in duplicate at 25 °C. The concentrations of NAD^+, $NADP^+$, and G6P were measured enzymatically with G6PD. Data were analyzed on an IBM computer using the equations for kinetic mechanisms and inhibition patterns developed by Cleland (1979).

Polyacrylamide gel electrophoresis

SDS-denaturing gel electrophoresis was done by the method of Laemmli (1970) and gels were silver stained using the nondiamine method of Merril (1990).

[1] Treatment with $MgCl_2$ was found to be necessary for disrupting the large, gelatinous residue that contained a substantial proportion of the G6PD. The residue could also be disrupted by treatment with DNase. No such residue was seen when extracts were prepared from uninfected SU294 cells or cells infected with plasmid lacking a pLmz insert. It may, therefore, result from interaction between *L. mesenteroides* G6PD and plasmid DNA. Poteete et al. (1991) previously found that the addition of $MgCl_2$ and DNase I helped decrease viscosity in cells that overexpress lysozyme. We found that the addition of $MgCl_2$ alone was sufficient to decrease the viscosity present after the cells were lysed.

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