Delineation of the Roles of Amino Acids Involved in the Catalytic Functions of *Leuconostoc mesenteroides* Glucose 6-Phosphate Dehydrogenase†

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ABSTRACT: The roles of particular amino acids in substrate and coenzyme binding and catalysis of glucose-6-phosphate dehydrogenase of *Leuconostoc mesenteroides* have been investigated by site-directed mutagenesis, kinetic analysis, and determination of binding constants. The enzyme from this species has functional dual $NADP^+/NAD^+$ specificity. Previous investigations in our laboratories determined the three-dimensional structure**.** Kinetic studies showed an ordered mechanism for the NADP-linked reaction while the NAD-linked reaction is random. His-240 was identified as the catalytic base, and Arg-46 was identified as important for $NADP^+$ but not NAD^+ binding. Mutations have been selected on the basis of the three-dimensional structure. Kinetic studies of 14 mutant enzymes are reported and kinetic mechanisms are reported for 5 mutant enzymes. Fourteen substrate or coenzyme dissociation constants have been measured for 11 mutant enzymes. Roles of particular residues are inferred from k_{cat}, K_m, k_{cat}/K_m, K_d, and changes in kinetic mechanism. Results for enzymes K182R, K182Q, K343R, and K343Q establish Lys-182 and Lys-343 as important in binding substrate both to free enzyme and during catalysis. Studies of mutant enzymes Y415F and Y179F showed no significant contribution for Tyr-415 to substrate binding and only a small contribution for Tyr-179. Changes in kinetics for T14A, Q47E, and R46A enzymes implicate these residues, to differing extents, in coenzyme binding and discrimination between $NADP^+$ and NAD^+. By the same measure, Lys-343 is also involved in defining coenzyme specificity. Decrease in k_{cat} and k_{cat}/K_m for the D374Q mutant enzyme defines the way Asp-374, unique to *L. mesenteroides* G6PD, modulates stabilization of the enzyme during catalysis by its interaction with Lys-182. The greatly reduced k_{cat} values of enzymes P149V and P149G indicate the importance of the cis conformation of Pro-149 in accessing the correct transition state.

Glucose 6-phosphate dehydrogenase (G6PD)[1] from most organisms utilizes $NADP^+$ as its coenzyme to oxidize glucose 6-phosphate (G6P). G6PD from *Leuconostoc mesenteroides*, however, can utilize either $NADP^+$ or NAD^+ (*1*), and this unusual dual coenzyme specificity plays an essential role in the metabolism of the bacteria (*2*). The enzyme's amino acid sequence (*3*) shows substantial homology with those of the 34 other G6PDs that have been sequenced (*4*). It is of interest to establish the roles of those amino acids in the *L. mesenteroides* enzyme that have functions common to all G6PDs, as well as those that play specific roles required for its unique catalytic properties. A catalytic mechanism has been proposed for *L. mesenteroides* G6PD in which His-240 acts as the general base, assisted by Asp-177 (*5*). Both of these residues are conserved and the mechanism can, therefore, be generalized to all G6PDs. Arg-46 was shown to play an important role in binding $NADP^+$ but not NAD^+ (*6*). This arginine residue is also conserved in all G6PDs, and its role in $NADP^+$ binding is, therefore, presumed to be universal.

From the three-dimensional structure of the *L. mesenteroides* G6PD apo-enzyme crystallized from phosphate (Figure 1a), now designated a phosphate inhibitor complex, the substrate binding site was inferred from the juxtaposition of the "inner" phosphate ion (i.e., more internal in the structure), which is bound to both subunits, and the eight-residue peptide conserved in all G6PDs (*7*). This suggested that the side chains of Glu-147, Lys-148, His-178 (through its Nδ1 position), and Tyr-415 could interact with the phosphate moiety of the substrate. A second, "outer" phosphate ion (i.e., nearer to the enzyme surface) in the structure of the enzyme−phosphate complex, bound to only one of the dimeric enzyme's subunits, also interacts with His-178 (through its Nϵ2 position), as well as with Tyr-179, Lys-182, and Lys-343. His-178 is part of the conserved peptide, and its role in binding the phosphate moiety of the substrate has been confirmed using site-directed mutagenesis (*5*). The conserved His-240 was seen to be in the proposed active site (*7*). Further support for the position of G6P in the active site came from

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[1] Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; G6P, glucose 6-phosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; S−NAD^+ and S−$NADP^+$, thionicotinamide analogues of NAD^+ and $NADP^+$, respectively.

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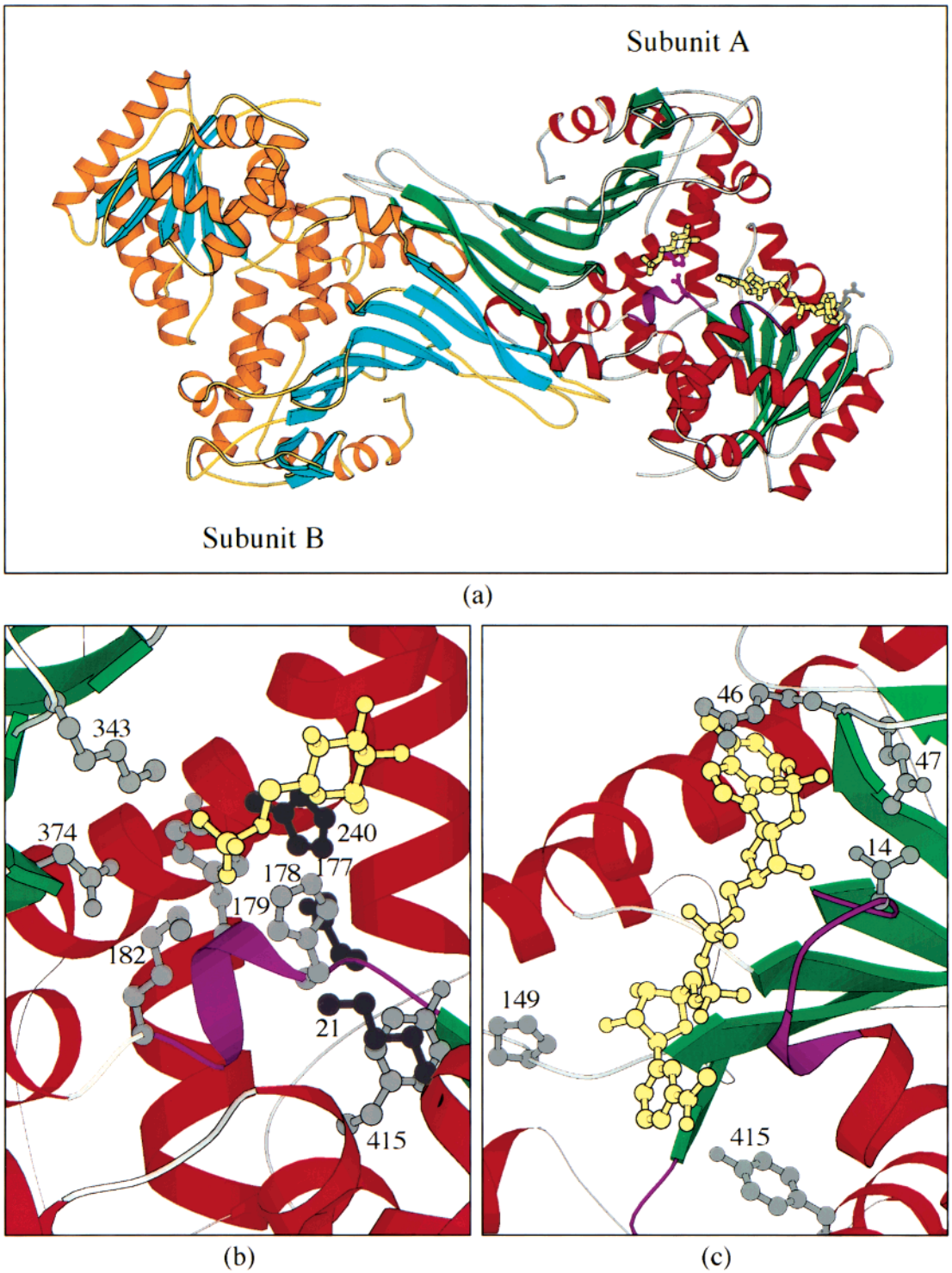

FIGURE 1: (a) The *Leuconostoc mesenteroides* G6PD dimer. In subunit A, helices are red and the two sheets green; in subunit B, helices are orange and sheets cyan. The coenzyme NADP and the substrate G6P are shown in ball-and-stick representation (in cream, outlined in black) as they bind in their binary complexes (*8*).[2] Close to the coenzyme, the stretch of main chain colored purple is the dinucleotide binding fingerprint GXXGDLA, residues 12−18 in *L. mesenteroides* G6PD. Arg-46, which interacts with the 2′-phosphate of $NADP^+$ is drawn in ball-and-stick. The stretch of main chain colored purple near the substrate is the conserved peptide RIDHYLGK, residues 175−182 in *L. mesenteroides* G6PD. Residues Asp-177 and His-240, the catalytic base, are drawn in ball-and-stick (purple). Figure 1 was prepared using BOBSCRIPT (*26*, *27*). (b) Region close to G6P. Residues discussed in the text are drawn in ball-and-stick; the His-Asp pair, residues 240 and 177, are black. His-178, Tyr-179, Lys-182, and Lys-343 are seen to interact with the 6-phosphate of G6P. Asp-374 is positioned to interact with Lys-182. Tyr-415 and Lys-21 (black) are seen to be more distant from G6P. The orientation and color scheme are as in part a. (c) Region close to the coenzyme, shown with $NADP^+$ present. Thr-14, a part of the dinucleotide binding fingerprint, Arg-46 and Gln-47, drawn in ball-and-stick, are seen close to the adenosine moiety. Pro-149, which may be cis or trans, is drawn and Tyr 415 is also shown. The color scheme is as in the previous figures, but the view is rotated by approximately 90° for clarity.

the elucidation of the catalytic mechanism, which requires that the Nϵ2 atom of His-240 be in proximity to the C1−OH of the substrate (*5*). Recently, the structures of the G6P−enzyme (Q365C) and NADPH−G6P enzyme (D177N)

Table 1: Oligonucleotide Sequences

enzyme	sequence of oligonucleotide[a]
T14A	CTTTCTTTGGTGGC**G**CTGGTGACTTGGCC
T14S	CTTTGGTGGC**T**CTGGTGACTTGG
R46E	GTTGGAACGGCC**GAA**CAAGCCCTCAATG
Q47A	GGAACGGCCCGT**GC**AGCCCTCAATGATG
Q47E	GGAACGGCCCGT**G**AAGCCCTCAATGATC
P149G	GATGATTGAAAAG**GG**TTTCGGTACATC
P149V	GATGATTGAAAAG**GTG**TTCGGTACATC
Y179F	CCGTATTGACCACT**T**CCTTGGTAAGG
K182Q	CCTTGGT**C**AGGAAATGG
K182R	CCACTACCTTGGT**CGT**GAAATGGTTCAAAAC
K343Q	GCGCTTAGCTGCT**C**A**G**CAGACACGGG
K343R	GCGCTTAGCTGCT**CGT**CAGACACGGG
D374Q	GCTGTCTTGTCAATTATCATT**C**A**G**CCAAAGGGTGCTATCG
Y415F	GCCAGAACCAT**T**CGAACGTATG

[a] Mutations to change amino acids in boldface.

complexes were solved (*8*), demonstrating that the phosphate moiety of the substrate binds to His-178, not via its Nδ1 atom, as expected, but via its Nϵ2 atom. On the basis of these structures, it appeared that Tyr-179 and Lys-182, both of which are part of the conserved peptide, and Lys-343, which is lysine or arginine in 77% of known sequences, also participate in binding the phosphate moiety of G6P (Figure 1b). Affinity labeling studies identified Lys-343 and Lys-21 as G6P binding residues (*9*), and this was confirmed for Lys-21 (*10*). Lys-21 is conserved in all but one G6PD (4).

L. mesenteroides G6PD has an aspartate residue at position 374; all other G6PDs, except that from *Leuconostoc mesenteroides dextranicus*, have a glutamine at the corresponding position in their sequences. The side chain of Asp-374 interacts with the ζ-amino group of Lys-182 in all *L. mesenteroides* G6PD structures that have been examined. In human G6PD, the glutamine that corresponds to Asp-374 interacts with Lys-205, which corresponds to Lys-182 in the *L. mesenteroides* enzyme (*11*). Pro-149 is conserved in all G6PDs. The crystal structure of the *L. mesenteroides* G6PD phosphate inhibitor complex shows that Pro-149 is cis in one subunit and trans in the other (*7*). Both Pro-149 residues are cis, however, in *L. mesenteroides* G6PD crystallized under other conditions whether as free enzyme or complexed with coenzyme, substrate, or both.[2]

The X-ray structures of the G6PD ternary complex (*8*) and coenzyme binary complexes[2] indicate involvement of Arg-46, Gln-47, and Thr-14 in coenzyme binding (Figure 1c)**.** The guanidinium of Arg 46 makes hydrogen bonds to the 2′-phosphate of $NADP^+$ and NADPH but does not interact with NAD^+. Gln-47 amide makes a direct hydrogen bond to an adenine ribose hydroxyl of NAD^+ and the 2′-phosphate of NADPH, and an indirect one through an ordered water to $NADP^+$. Thr 14 Oγ1 makes a hydrogen bond to the adenine ribose 3′-OH in all three complexes. Gln-47 is unique in the *L. mesenteroides* enzyme, and Thr-14 is not conserved (but the replacement is conservative, serine for threonine in all but two sequences).

In this paper we describe experiments utilizing site-directed mutagenesis to change some of these amino acid residues and kinetic and binding studies to delineate their roles in the catalytic functions of G6PDs in general and of *L. mesenteroides* G6PD in particular.

[2] Naylor et al. Manuscript in preparation.

EXPERIMENTAL PROCEDURES

Materials. NAD^+ and $NADP^+$ were obtained from Boehringer-Mannheim; NADH, NADPH, S−NAD^+, S−$NADP^+$, G6P, Reactive Green-19 agarose, lysozyme, and Protease-X (thermolysin) were obtained from Sigma; Matrex gels Purple A and Orange B, and Centriflo CF-50 membrane cones were obtained from Amicon Corp.; Coomassie protein assay reagent and bovine serum albumin standard were obtained from Pierce; shrimp alkaline phosphatase and the Sequenase version 2.0 DNA sequencing system were from Amersham International plc; a Chameleon site-directed mutagenesis kit was obtained from Stratagene; pUC-19 and M-13 bacteriophage were obtained from Gibco-BRL; a Wizard miniprep DNA purification kit was obtained from Fisher Scientific; restriction endonucleases, T4 polynucleotide kinase, and DNA ligase were obtained from New England Biolabs; and α-^{35}S dATP was obtained from New England Nuclear. Monoclonal antibody was generously supplied by Dr. Mark Levy of Syva Company, Palo Alto, CA. Oligonucleotides were synthesized by Genosys. The sequences for the various mutations are indicated in Table 1, where changes from wild-type sequences are underlined.

DNA Techniques. DNA techniques followed the standard procedures described by Sambrook et al. (*12*) and as previously reported (*6*). Site-directed mutagenesis was performed with the Chameleon site-directed mutagenesis kit using the oligonucleotides listed in Table 1. Proteins were expressed in *Escherichia coli* strain SU294, which lacks the G6PD gene (*10*). DNA was prepared from the transformed bacteria, and to ensure that no other mutations had been introduced, the entire insert was sequenced either in our laboratory or commercially at the Biotechnology Resource Center, Cornell University, Ithaca, NY.

Enzyme Isolation and Assay. Unless noted, wild-type and mutant enzymes were isolated by the procedures described previously (*6*, *10*). T14A G6PD could not be eluted from the Matrex Gel Orange column with $NADP^+$; instead, it was eluted with 1.0 M KCl, concentrated with a CF-50 Centriflo membrane cone, and dialyzed extensively against 50 mM Tris-HCl, pH 7.8. R46E G6PD did not adhere to the Matrex Gel Orange column. It was diluted so that the Tris-HCl concentration was 5 mM, and $MgCl_2$ was added to 10 mM. The enzyme was placed on a Reactive Green-19 agarose column, eluted with 1.0 M KCl, and dialyzed against 50 mM

Tris-HCl, pH 7.8. Q47E G6PD bound weakly to the Matrex Gel Orange column and was eluted with the application buffer, 50 mM Tris-HCl, pH 7.8, without $NADP^+$. All enzymes were judged to be homogeneous by SDS−PAGE, using 5−10 μg of protein, and silver staining (*13*). Routine assays for G6PD were performed at 25 °C by monitoring the production of NADPH in a Gilford 240 spectrophotometer at 340 nm. Reactions were initiated by the addition of enzyme to 33 mM Tris-HCl, pH 7.6, containing 2.28 mM G6P and 0.160 mM $NADP^+$. During their purification, mutant enzymes with low catalytic activity (mutations P149V and P149G) were detected on SDS−PAGE gels with silver staining (*12*) and confirmed by western blotting using a monoclonal antibody prepared against native *L. mesenteroides* G6PD (*14*). Protein concentrations were routinely determined using the Coomassie reagent, with bovine serum albumin as the standard (*15*). These values were corrected on the basis of the known extinction coefficient of wild-type enzyme at 280.5 nm, assuming that the mutations did not alter the extinction coefficients of the mutant enzymes (*5*).

Kinetics. Kinetic constants were determined at 25 °C as described previously (*6*). Briefly, this involved determining apparent kinetic constants initially and then, using these values, selecting five concentrations each of coenzyme and substrate for the determination of the true kinetic constants. Coenzyme and substrate concentrations were determined enzymatically, and the pH values of the reaction mixtures were measured after the assays were completed. In those instances where the reactions did not obey Michaelis−Menten kinetics, apparent K_m values were determined using saturating concentrations of coenzyme with a range of 7 or more substrate concentrations and vice versa. Apparent k_{cat} values were derived from the mean of the apparent V_{max} values associated with each of the apparent K_m values. Inhibition studies with reduced coenzymes were undertaken with those mutant enzymes with significantly elevated $NADP^+$ K_m values and some which did not show hyperbolic kinetics. Such studies were conducted at near-saturating concentration of substrate, varying the concentration of coenzyme, or vice versa, in the absence and in the presence of two concentrations of inhibitor. Data were analyzed using the following computer programs (*16*): HYPER for apparent kinetic constants, SEQUEN for true kinetic constants, and COMP and NONCOMP to analyze inhibition data.

Dissociation Constants. Dissociation constants were determined by measuring the protection afforded by G6P, NAD^+, or $NADP^+$ against thermolysin inactivation at 40 °C, as previously described (*17*). Appropriate concentrations of the mutant enzymes were incubated at 40 °C with or without various concentrations of added ligand and thermolysin. Aliquots were removed at various times, diluted in ice-cold water, and assayed in duplicate or triplicate using a Gilford Response spectrophotometer. The kinetics of inactivation were first-order in all experiments. Percent protection was calculated for each substrate concentration, and the data were plotted in double-reciprocal form and analyzed as previously described (*17*) using Cleland's HYPER program.

Circular Dichroism Spectra. CD spectra of enzymes in 25 mM potassium phosphate containing 50 mM KCl, pH 7.8 were measured between 290 and 190 nm at room temperature in an Aviv CD spectrometer model 202 using a 1 mm cuvette.

Table 2: Conservation of Mutated Amino Acid Residues[a]

residue	sequence no.[b]	mutations	alternative residues (no. of occurrences)[c]
Thr	14	Ala, Ser	Ser (26), Thr (7), Lys (2)
Lys	21	Arg, Gln	Lys (34), Met (1)
Arg	46	Ala, Glu	conserved
Gln	47	Ala, Glu	Ser (18), Arg (6), Thr (6), Ala (2), Gln (2), Lys (1)
Pro	149	Gly, Val	conserved
His	178	Asn	conserved
Tyr	179	Phe	conserved
Lys	182	Arg, Gln	conserved
Lys	343	Arg, Gln	Arg (16), Lys (11), Gly (3), Gln (2), Asp (1), His (1), Ser (1)
Asp	374	Gln	Gln (32), Asp (2),[d] gap (1)
Tyr	415	Phe	conserved

[a] Based on 35 sequence alignments cited in Au et al. (*4*). [b] Sequence number in *L. mesenteroides* G6PD. [c] Residues found in corresponding position in other G6PDs. [d] Asp found only in *L. mesenteroides* and *L. mesenteroides dextranicus.*

RESULTS

Table 2 lists all the amino acid residues in *L. mesenteroides* G6PD mutated for these studies and their degree of conservation among known G6PD sequences. Kinetic constants for all the mutant enzymes are listed in Tables 3 (NADP reaction) and 4 (NAD reaction). Ligand dissociation constants and free energies of binding are listed in Table 5.

Kinetic and Dissociation Constants for the Lys-182, Lys-343, and Lys-21 Mutant Enzymes. To examine the role of Lys-182 and Lys-343 in G6P binding, we prepared mutant enzymes in which each of these lysine residues was replaced by arginine (to retain the positive charge) or glutamine (to retain its polar character). K182Q G6PD displays nonhyperbolic kinetics in the NAD-linked reaction when each of the G6P and NAD^+ concentrations was varied. Apparent kinetic constants for this reaction were determined at near-saturating concentrations of the nonvaried substrate, where the increase in velocity showed strictly hyperbolic dependence on varied substrate concentration. The principal effects of the substitution of arginine or glutamine for Lys-182 are the large increases (approximately 2 orders of magnitude) in K_m for G6P in both the NADP- and the NAD-linked reactions, indicating that Lys-182 plays an important role in substrate binding during catalysis. There are not large differences between kinetic constants for the K182R and K182Q enzymes, suggesting that, despite its positive charge, Arg-182 cannot substitute effectively for lysine in binding G6P in the enzyme's transition state complexes. The dissociation constant for G6P is increased significantly for both the K182Q and K182R enzymes, demonstrating that Lys-182 is also involved in binding G6P to free enzyme.

The K343Q enzyme displays K_m values for G6P that have increased more than 300-fold for the NAD-linked and that have increased nearly 1000-fold for the NADP-linked reaction over the corresponding values for wild-type enzyme. Nonhyperbolic kinetics were obtained for the NADP-linked reaction of the K343Q enzyme when each of the G6P and $NADP^+$ concentrations was varied. The apparent K_m for G6P was determined at saturating $NADP^+$ concentration, and the

Table 3: Kinetic Constants for Mutant G6PDs, NADP Reaction

enzyme	K_m G6P (μM)	K_m $NADP^+$ (μM)	K_i $NADP^+$ (μM)[a]	k_{cat} (min^{-1})	k_{cat}/K_m G6P ($min^{-1}\,\mu M^{-1}$)	k_{cat}/K_m $NADP^+$ ($min^{-1}\,\mu M^{-1}$)
W.T.[b]	114 ± 11	8.0 ± 0.7	3.4 ± 1.0	20 200[c]	178[c]	2530[c]
T14A	378 ± 52	86 ± 12	169 ± 34	37 900	100	441
T14S	118 ± 8.2	14 ± 1.0	16 ± 2.2	22 700	192	1620
K21Q[d]	6070 ± 575	7.3 ± 1.1	7.8 ± 1.6	14 100[c]	2.3[c]	1930[c]
K21R[d]	239 ± 18	8.1 ± 0.5	6.2 ± 1.2	22 500[c]	94[c]	2780[c]
R46E[e]	1040 ± 121	21 000 ± 1 300	ND[f]	38 000	37	1.8
Q47A[e]	75 ± 6.1	7.5 ± 0.8	ND[f]	14 100	188	1880
Q47E	239 ± 14	84 ± 5.3	87 ± 8.6	33 100	138	394
P149G	2600 ± 167	24 ± 1.4	19 ± 3.0	512	0.20	21
P149V	1080 ± 157	18 ± 3.0	34 ± 7.7	36	0.03	2.0
Y179F	441 ± 101	14 ± 3.3	7.3 ± 3.7	15 800	36	1130
K182Q	13 100 ± 2100	6.5 ± 1.2	7.8 ± 2.4	18 500	1.4	2800
K182R	16 100 ± 1700	15 ± 1.7	8.0 ± 2.8	35 100	2.2	2340
K343Q[e]	106 000 ± 16 000	46 ± 3.9	ND[f]	42 700	0.40	928
K343R	3080 ± 275	8.0 ± 0.8	6.7 ± 1.7	24 200	7.9	2030
D374Q	193 ± 36	13 ± 3.1	7.9 ± 3.2	4 510	23	346
Y415F	74 ± 7.5	6.3 ± 1.2	17 ± 3.4	11 000	149	1750

[a] In an ordered mechanism where the coenzyme binds first, such as the NADP-linked reaction of the wild-type *L. mesenteroides* G6PD, the K_i for that coenzyme represents its dissociation constant, but if that reaction mechanism is random, this K_i value has no physical significance (*25*). [b] W.T. = wild-type enzyme; data from Lee and Levy (*10*). [c] The corresponding values for these constants reported in Lee and Levy (*10*) and Levy et al. (*6*) were calculated using uncorrected protein concentrations (see Experimental Procedures). [d] Data from Lee and Levy *(10)*. [e] Apparent kinetic values. [f] ND = not determined.

Table 4. Kinetic Constants for Mutant G6PDs, NAD Reaction

enzyme	K_m G6P (μM)	K_m NAD^+ (μM)	k_{cat} (min^{-1})	k_{cat}/K_m G6P (min^{-1})	k_{cat}/K_m NAD^+ ($min^{-1}\,\mu M^{-1}$)
W.T.[a]	69 ± 9.0	162 ± 18	43 600[b]	630[b]	269[b]
T14A	169 ± 20	2340 ± 269	48 500	285	21
T14S	75 ± 11	199 ± 26	35 000	467	176
K21Q[c]	2500 ± 200	518 ± 72	55 100[b]	22[b]	106[b]
K21R[c]	97 ± 11	240 ± 26	42 600[b]	441[b]	178[b]
R46E	132 ± 11	986 ± 71	39 500	299	40
Q47A[d]	96 ± 3.6	234 ± 5.9	34 600	360	148
Q47E	233 ± 19	310 ± 26	48 500	208	156
P149G	825 ± 121	1760 ± 199	1 050	1.3	0.60
P149V	1730 ± 371	2230 ± 428	166	0.10	0.07
Y179F	165 ± 37	184 ± 26	28 500	173	155
K182Q[d]	6800 ± 490	82 ± 2.0	15 300	2.3	187
K182R	3670 ± 750	379 ± 120	64 100	17	169
K343Q	25 300 ± 6000	503 ± 130	51 000	2.0	101
K343R	1260 ± 385	338 ± 40	45 000	36	133
D374Q	135 ± 17	571 ± 60	12 500	93	22
Y415F	63 ± 7.8	116 ± 13	19 000	302	164

[a] W.T. = wild-type enzyme; data from Lee and Levy (*10*). [b] The corresponding values for these constants reported in Lee and Levy (*10*) and Levy et al. (*6*) were calculated using uncorrected protein concentrations (see Experimental Procedures). [c] Data from Lee and Levy *(10)*. [d] Apparent kinetic values.

apparent K_m for $NADP^+$ was determined using 1.0 M G6P. In both instances the kinetics were strictly hyperbolic. The K_d for G6P for this mutant enzyme is also increased substantially. For the K343R mutant enzyme, the K_m and K_d values for G6P are also increased and no other significant changes were noted for measured constants. In contrast to the results with the K182Q and K182R mutant enzymes, the substitution of arginine for lysine at position 343 has a much smaller effect on the properties of the enzyme than the substitution of glutamine. Lys-343, like Lys-182, plays a key role in binding the phosphate moiety of G6P to free enzyme and during catalysis, but there seems to be greater flexibility in this interaction, enabling an Arg residue to serve the same function, though less efficiently.

In addition to their major effects on G6P binding to free enzyme and during catalysis, some more subtle effects are evident in these mutants. The NADP-linked reaction of the K343Q enzyme and the NAD-linked reaction of the K182Q enzyme both show nonhyperbolic kinetics. The apparent coenzyme K_m and k_{cat} values for the former are higher, and for the latter they are lower, than the corresponding kinetic constants for wild-type enzyme. The significance of these findings is discussed below.

When Lys-21 was changed to glutamine, the principal effects were large increases in the K_m values for G6P and corresponding reductions in the values for k_{cat}/K_m for G6P (*10*). The K21R mutant behaves similarly to wild-type enzyme. The dissociation constant for G6P in the K21Q enzyme is only increased 2-fold, and that of the K21R enzyme is not significantly altered, compared to the corresponding K_d values in wild-type enzyme (Table 5). Unlike Lys-182 and Lys-343, Lys-21 is not involved significantly in binding G6P to free enzyme but does play a role in binding substrate during catalysis.

Kinetic Constants for the Tyr-179 and Tyr-415 Mutant Enzymes. The possible contributions of Tyr-179 and Tyr-

Table 5: Dissociation Constants and Apparent Free Energies of Ligand Binding to Enzyme for Mutant G6PDs

	enzyme	K_d (mM)	ΔG^o (kcal/mol)	$\Delta\Delta G^o$ (kcal/mol)[c]
a. G6P	W.T.[a]	1.2	4.0	
	T14A	1.1 ± 0.1	4.0	0
	K21Q	2.5 ± 0.5	3.5	0.5
	K21R	1.3 ± 0.1	3.9	0.1
	Q47E	2.6 ± 1.0	3.5	0.5
	P149G	7.5 ± 0.9	2.9	1.1
	P149V	5.9 ± 0.3	3.0	1.0
	H178N[b]	12.5 ± 2.1	2.6	1.4
	K182Q	25.0 ± 0.5	2.2	1.8
	K182R	10.2 ± 0.6	2.7	1.3
	K343Q	12.7 ± 2.4	2.6	1.4
	K343R	4.4 ± 0.3	3.2	0.8
	Y415F	2.6 ± 0.3	3.5	0.5
b. $NADP^+$	W.T.[a]	0.0061	7.1	
	T14A	0.48 ± 0.06	4.5	2.6
	Q47E	0.12 ± 0.05	5.3	1.8
c. NAD^+	W.T.[a]	3.8	3.3	
	Q47E	3.2 ± 0.4	3.4	0.1

[a] W.T. = wild-type enzyme; data from Kurlandsky et al. (*17*). [b] From Cosgrove et al.(*5*). [c] Difference between ΔG^o values of mutant and wild-type enzymes.

415 to substrate binding were examined by preparing the respective phenylalanine mutant enzymes, Y179F and Y415F. Compared to the lysine residues described above, these tyrosine residues play minor roles in binding G6P. The Y179F enzyme displays slightly elevated K_m values for G6P and diminished k_{cat} values in both the NADP- and the NAD-linked reactions, resulting in approximately 5-fold reduced k_{cat}/K_m values for G6P. The Y415F mutant displays slightly reduced k_{cat} values in both reactions and a somewhat higher K_i value for $NADP^+$ but no significant effects on the K_m values for G6P. The K_d for G6P is only slightly increased. Tyr-415 is not involved in binding G6P to the enzyme.

Kinetic Constants for the Thr-14, Gln-47, and Arg-46 Mutant Enzymes. Structural studies implicate Thr-14, Gln-47, and Arg-46 in coenzyme binding (Figure 1c). To elucidate their differential role in binding $NADP^+$ and NAD^+, we replaced these residues with amino acids that might not be able to interact with one or the other coenzyme. Substitution with alanine should prevent such interaction, and the results, in agreement with the prediction for the R46A mutant, have been published (*6*). The T14S mutant enzyme was generated because most G6PDs, including the human enzyme, have a serine in the corresponding location and to establish whether the more hydrophobic threonine side chain plays a role in coenzyme binding to *L. mesenteroides* G6PD. We replaced each of the amino acids Arg-46 and Gln-47 with glutamate in an attempt to create a negatively charged local environment that might favor the binding of NAD^+ over $NADP^+$.

The greatest effect among these mutant enzymes is the large increase in the K_m for $NADP^+$ shown by the R46E mutant enzyme. We were unable to determine true kinetic constants for the NADP-linked reaction catalyzed by R46E G6PD because the kinetics are nonhyperbolic. From the apparent K_m for $NADP^+$ and the K_m for NAD^+ in this mutant enzyme it is clear that, as expected, replacement of the positively charged arginine residue with a negatively charged glutamate causes a very large reduction in $NADP^+$ binding and a comparatively small reduction in NAD^+ binding during catalysis.

When Thr-14 is replaced by alanine, there is a substantial diminution in the binding of both coenzymes during catalysis. The dissociation constant for $NADP^+$ for the T14A enzyme is increased by 2 orders of magnitude over that for the wild-type enzyme. Replacing Thr-14 with a serine residue has little effect on any kinetic constant.

The apparent kinetic constants for the Q47A enzyme are not substantially altered in either the NADP- or the NAD-linked reaction. This enzyme does not yield hyperbolic kinetics for either reaction. The effects of the Q47E mutation on the kinetic constants in the NAD-linked reaction are small, as are the effects on K_d for NAD^+. In the NADP-linked reaction, the apparent K_m value for $NADP^+$ is increased 10-fold over the K_m for the wild-type enzyme, and k_{cat} is also increased significantly; the K_d for $NADP^+$ is increased 20-fold.

Kinetic Mechanisms. The kinetic mechanisms of the NADP- and NAD-linked reactions are ordered ($NADP^+$ binds before G6P) and steady-state random, respectively (*18*). We examined the kinetic mechanisms of several mutant G6PDs with perturbed interactions with coenzyme or substrate, as well as the wild-type recombinant enzyme. The results are shown in Table 6. The recombinant wild-type enzyme shows $NADP^+$-competitive inhibition by NADPH and NAD^+-noncompetitive inhibition by NADH. This behavior is consistent with an ordered mechanism, with coenzyme binding first for the NADP-linked and a random mechanism for the NAD-linked reaction; as expected, this behavior is identical with that of the wild-type G6PD isolated from *L. mesenteroides* (*18*, *19*). For T14A G6PD, NADPH inhibits noncompetitively with respect to $NADP^+$. This suggests that the reaction proceeds via a random mechanism. Both the K_m and K_d for $NADP^+$ in this enzyme are increased substantially over the corresponding values for the wild-type enzyme. The K_m for $NADP^+$ for R46A G6PD is so high (*6*) that it is impractical to test the NADP-linked reaction for NADPH inhibition. As the thionicotinamide analogues, $S-NADP^+$ and $S-NAD^+$, have substantially lower K_m values than the corresponding natural coenzymes, while the kinetic mechanisms for the reactions in which they participate remain unaltered (*20*), we examined NADPH inhibition with respect to $S-NADP^+$ with R46A G6PD and found that it is noncompetitive. Likewise, both S−NADH and NADH inhibit noncompetitively with respect to NAD^+, consistent with the NAD-linked reaction mechanism remaining random. The Q47E mutant enzyme, likewise, displays random kinetics for both reactions. The T14A, R46A, and Q47E mutant enzymes all display weakened $NADP^+$ binding during catalysis, and for each one the kinetic mechanism of the NADP-linked reaction is random.

The kinetic mechanisms of the NAD-linked reaction for K182Q G6PD and the NADP-linked reaction for the K343Q enzyme were examined in the same manner. NADH inhibition of the former is noncompetitive with respect to NAD^+, and NADPH inhibition of the NADP-linked reaction of the K343Q enzyme is also noncompetitive. The significance of these findings is discussed below.

Kinetic Constants for the Pro-149 Mutant Enzymes. P149V and P149G mutant enzymes were prepared in order to examine the significance of the alternative cis and trans

Table 6: Kinetic Mechanisms

enzyme	varied ligand	apparent K_m (μM)	inhibitor	inhibition[b]	K_{is} (μM)	K_{ii} (μM)
W.T.[a]	$NADP^+$	7.7 ± 0.45	NADPH	competitive	31 ± 2.4	
W.T.	NAD^+	224 ± 27	NADH	noncompetitive	827 ± 294	1590 ± 346
T14A	$NADP^+$	122 ± 25	NADPH	noncompetitive	247 ± 88	1230 ± 526
R46A	S−$NADP^+$	2640 ± 674	NADPH	noncompetitive	37 000 ± 16 400	18 000 ± 7290
R46A	NAD^+	1130 ± 322	NADH	noncompetitive	2020 ± 1600	1870 ± 524
R46A	S−NAD^+	222 ± 21	NADH	noncompetitive	10 500 ± 1720	33 200 ± 10 600
Q47E	$NADP^+$	54 ± 11	NADPH	noncompetitive	351 ± 13	1370 ± 340
Q47E	NAD^+	236 ± 59	NADH	noncompetitive	753 ± 430	1720 ± 464
K182Q	NAD^+	155 ± 21	NADH	noncompetitive	1290 ± 750	1130 ± 210
K343Q	$NADP^+$	51 ± 10	NADPH	noncompetitive	151 ± 46	2320 ± 1600

[a] W.T. = wild type. [b] Data were fit to both COMP and NONCOMP programs *(16)*, and the assignment was based on which pattern gave the better standard error as well as the better standard errors for kinetic constants.

conformations of Pro-149 on catalysis.[2] There are large reductions in k_{cat} values for both the NAD- and the NADP-linked reactions in both mutant enzymes and also significant increases in G6P K_m values for both reactions and in the K_m for NAD^+. The corresponding k_{cat}/K_m values are reduced by 2−3 orders of magnitude. These effects are greater with P149V G6PD than with the P149G enzyme. The K_d for G6P is increased significantly for both mutant enzymes.

Characteristics of the D374Q Enzyme. Because Asp-374 is unique in *L. mesenteroides* G6PD and interacts with Lys-182 (Figure 1b), it seemed possible that this residue might play an important role associated with one of this enzyme's characteristic functions. It was replaced with glutamine, the amino acid found in all other G6PDs at this location. The principal effects of this mutation on kinetic constants are significant decreases in k_{cat} and k_{cat}/K_m values in both reactions. The D374Q G6PD is also substantially less stable than wild-type enzyme. After incubating at 40 °C, D374Q G6PD retained only 37% of its activity after 2 min and 16% after 30 min, whereas wild-type enzyme retained 97% and 95% of its activity, respectively. When the D374Q enzyme was heated for 5 min at 40 °C in the absence and presence of substrate or coenzyme, each at $20K_m$ concentration, G6P provided 86% protection, NAD^+ protected 29%, and $NADP^+$ protected 31%. Nondenaturing polyacrylamide gels support the view that the D374Q enzyme dimers dissociate upon heating (C. Wojdyla, unpublished).

CD Spectra. The CD spectra of the wild-type enzyme and enzymes exhibiting large changes in kinetic or binding constants (from mutants T14A, K21Q, R46E, P149G, P149V, K182Q, K343Q, and D374Q) were very similar (data not shown). The wild-type enzyme shows broad ellipticity minima at 218.5 and 210.5 nm, consistent with the fact that 192 of the enzyme's 485 amino acid residues are contained in α-helices and 93 residues in β-strands *(7)*. All the mutant enzymes showed CD spectra that are very similar to that of the wild-type enzyme, indicating that none of these mutations caused general conformational changes.

DISCUSSION

The Substrate Binding Site. The location of the substrate binding site between the coenzyme binding domain and the larger β + α domain was inferred from X-ray structural studies of the wild-type *L. mesenteroides* G6PD−phosphate complex (*7*). In this structure, the enzyme contains an inner phosphate ion bound to each subunit at the Nδ1 position of His-178 and an outer phosphate ion bound to the Nϵ2 position of the same His-178 in only one of the two subunits. We have confirmed the important role for His-178 in binding the phosphate moiety of G6P in the transition state complex from kinetic studies of the H178N mutant enzyme (*5*). The X-ray structures of the *L. mesenteroides* G6P enzyme (Q365C) and NADPH−G6P enzyme (D177N) complexes confirm the location of the bound G6P close to His-240 and His-178, in the pocket between domains. They show, however, that the phosphate moiety of G6P is bound through the Nϵ2 position of His-178, not the Nδ1 site (*8*). These structures also show interactions between the phosphate group of the substrate and Lys-182, Lys-343, and Tyr-179 (Figure 1b).

Previous experiments implicated three lysine residues in substrate binding. Roles for Lys-21 and Lys-343 were inferred from their identification as substrate binding residues through affinity labeling studies (*8*), and this was confirmed for Lys-21 by the large, selective effect on the G6P K_m in the K21Q mutant enzyme (*10*). Lys-182 and Lys-343, but not Lys-21, interact with the substrate's phosphate moiety in both binary and ternary complexes (*8*). The properties of the enzymes with mutations at positions 21, 182, and 343 reported here suggest that these three lysine residues play different roles in *L. mesenteroides* G6PD. Lys-21 makes only a minor contribution to binding G6P to free enzyme, whereas both Lys-182 and Lys-343 play major roles, comparable to that of His-178. All three lysine residues make substantial contributions to binding the substrate during catalysis. The potential roles of Lys-182 and Lys-343 in the dual coenzyme specificity of the enzyme are discussed below.

On the basis of the structures of both G6P-containing complexes of *L. mesenteroides* G6PD, it was expected that Tyr-179 would play a significant role in binding G6P: the Tyr-OH is positioned to make an H-bond with one of the phosphate oxygen atoms (*8*). For both the NADP- and NAD-linked reactions, however, the Y179F mutant enzyme shows only small increases in K_m values for G6P and decreases in k_{cat} values, leading to modestly reduced k_{cat}/K_m values for G6P. During catalysis in both the NADP- and the NAD-linked reactions, therefore, the hydroxyl group of Tyr-179 makes a relatively small contribution to substrate binding.

The Coenzyme Binding Site. The X-ray structure of *L. mesenteroides* G6PD *(7)* and its coenzyme complexes implicate Thr-14, Arg-46, and Gln-47 in coenzyme binding (Figure 1c).[2] The important role that the positively charged

guanidinium of Arg-46 plays in selectively binding the 2′-adenosine phosphate of $NADP^+$ has been reported (*6*). In the R46E mutant enzyme, the negatively charged side chain causes a dramatic increase in the apparent K_m for $NADP^+$. This mutation also interferes, somewhat, with the binding of G6P in the NADP-linked reaction, as well as with NAD^+ in the NAD-linked reaction. The strong interaction of the Arg-46 residue with the adenosine 2′-phosphate of $NADP^+$, involving two hydrogen bonds between two guanidinium nitrogen atoms and two phosphate oxygen atoms (*6*), is probably the principal reason for the tighter binding of this coenzyme to the wild-type enzyme compared to NAD^+ binding (*16*).

X-ray structural studies reveal that Thr-14, in addition to binding the 2′-adenosine phosphate of $NADP^+$, participates directly in the binding of both coenzymes via a hydrogen bond from its side chain to the O3 atom of the adenosine ribose.[2] As anticipated, this function can be fulfilled by serine in the T14S enzyme with no significant change in kinetic constants (except for K_i for $NADP^+$) suggesting that the more hydrophobic nature of the threonine side chain does not contribute significantly to stabilizing coenzyme in the site. Substituting an alanine for Thr-14 causes a large increase in the dissociation constant for $NADP^+$ and increases in the coenzyme K_m for both $NADP^+$ and NAD^+.

The Gln-47 residue is unique in *L. mesenteroides* G6PD: most G6PDs, including the human enzyme, have a serine, and other G6PDs have threonine, arginine, or alanine residues at this position (*7*). Gln-47 is positioned to interact with either the 2′-phosphate of $NADP^+$ or the 2′-adenosine hydroxyl of NAD^+ in the *L. mesenteroides* enzyme (*7*), suggesting that it might play a role in the enzyme's dual coenzyme specificity. The X-ray structures of the enzyme−coenzyme complexes show that NAD^+ fails to order Arg-46 and allows Gln-47 to interact with the adenosine 2′-hydroxyl group through a direct hydrogen bond.[2] This interaction could not occur in Q47A G6PD. Solely on the basis of the magnitude of the apparent kinetic constants of the Q47A enzyme, none of which differ significantly from the corresponding values of the wild-type enzyme, it would appear that Gln-47 plays only a minor role in the energetics of coenzyme binding. However, this enzyme did not yield hyperbolic kinetics for either the NADP- or the NAD-linked reaction under conditions normally used to obtain kinetic constants. At saturating concentrations of either substrate or coenzyme, hyperbolic kinetics were observed. This suggests that Gln-47 may help to position the coenzyme correctly with respect to the substrate during catalysis. The Q47E mutant enzyme shows substantial increases in the K_d, K_m, and K_i values for $NADP^+$, but this may be a secondary effect due to the interaction between the glutamate residue with the neighboring Arg-46, thereby diminishing the effectiveness of that residue in interacting with the adenosine 2′-phosphate of $NADP^+$.

Dual Coenzyme Specificity and Catalytic Mechanism. *L. mesenteroides* G6PD can utilize either $NADP^+$ or NAD^+ to oxidize G6P. This unusual dual coenzyme specificity is accompanied by two other catalytic features: higher k_{cat} and coenzyme K_m values for the NAD-linked than the NADP-linked reaction (*10*) and different kinetic mechanisms, ordered for the NADP-linked and steady-state random for the NAD-linked reactions (*18*). This difference in kinetic mechanisms is the basis for an unusual regulatory feature of the enzyme, whereby NADPH and G6P can regulate the enzyme's selection of coenzyme utilization (*20*, *21*). The present investigation provides some insights into these characteristics of the enzyme.

Table 7: Catalytic Efficiencies of G6PD Mutants Involved in Coenzyme Binding[a]

	NADP reaction		NAD reaction	
enzyme	k_{cat}/K_A	$k_{cat}/K_{iA}K_B$	k_{cat}/K_A	$k_{cat}/K_{iA}K_B$
W.T.[b]	2530	52	269	1.1
R46A	13	0.018	57	0.11
T14A	441	0.59	21	0.06
Q47A	1180	ND[c]	148	ND[c]

[a] K_{iA} values for NAD reaction: wild type = 599 μM, R46A = 3230 μM, T14A = 4800 μM. Data for the wild-type, T14A, and Q47A enzymes are from Tables 2 and 3; data for the R46A enzyme are from Levy et al. (*6*). $K_A = K_m$ for coenzyme, $K_{iA} = K_i$ for coenzyme, $K_B = K_m$ for G6P. [b] W.T. = wild type. [c] ND = not determined. K_{iA} values not available from determination of apparent kinetic constants.

The catalytic efficiency of an enzyme is given by k_{cat}/K_m (*22*). When two substrates compete for the same enzyme, their specificity is determined by the ratios of their k_{cat}/K_m values (*24*). Table 7 lists the ratios of k_{cat} to the coenzyme K_m for the wild-type enzyme and mutant enzymes R46A, T14A, and Q47A. We reported earlier on the dramatic effect on coenzyme utilization in the R46A mutant enzyme, in which the efficiencies for the NADP- and NAD-linked reactions are reversed (*6*). Table 7 shows that, using this criterion for catalytic efficiency, the T14A mutation has a greater effect on the NAD-linked than the NADP-linked reaction and that the effect of the Q47A mutation is relatively small and approximately the same in both reactions. Both these mutant enzymes retain their greater efficiency with $NADP^+$. A more appropriate way of evaluating catalytic efficiency for a two-substrate enzyme that catalyzes a sequential mechanism, and that takes into account both substrates, is by the term $k_{cat}/K_{iA}K_B$, an expression that is proportional to the free energy of activation of the overall reaction (*23*). Calculations of this term for wild-type enzyme and the R46A and T14A mutant enzymes in Table 7 reinforce the conclusions about the effects of these mutations on the catalytic efficiencies of *L. mesenteroides* G6PD with $NADP^+$ and NAD^+. By comparing the $k_{cat}/K_{iA}K_B$ ratios for the NADP- and NAD-linked reactions, one can see that the specificity of the wild-type enzyme is 47 times greater for $NADP^+$ than for NAD^+ and that for T14A enzyme it is only 10-fold greater. The R46A enzyme has a 6-fold greater specificity for NAD^+. This calculation reinforces the critical role of Arg-46 in the enzyme's preference for $NADP^+$.

$NADP^+$ binds to wild-type enzyme some 200 times more tightly than G6P (*17*). The enzyme−G6P complex does not allow productive $NADP^+$ binding, yet it will allow NAD^+ to bind productively. Presumably, when G6P is bound, the high specificity of the enzyme's Arg-46 for the adenosine 2′-phosphate of $NADP^+$ (*6*) could prevent any reorientation of $NADP^+$, which might be required to form a productive ternary complex. The weaker interaction of the enzyme with NAD^+ could allow any such reorientation to proceed more readily. Likewise, the weakened interaction between the enzyme and $NADP^+$ in the R46A enzyme could permit such reorientation. In the T14A enzyme, binding to both coenzymes is weakened, and in the Q47E enzyme $NADP^+$

binding is selectively weakened by the introduction of a negative charge. The affinity for G6P to the T14A and Q47E mutant enzymes is not significantly altered (Table 5).

Inhibition by reduced coenzyme is competitive in the ordered NADP-linked mechanism and noncompetitive for the random NAD-linked mechanism (*18*, *19*). For each of the G6PDs from mutants T14A, R46A, and Q47E, NADPH inhibition of the NADP-linked reaction is noncompetitive (Table 5), consistent with a random mechanism. The K_m for $NADP^+$ is greatly increased for the T14A, R46A (*6*), and Q47E mutant enzymes. The k_{cat} values for the NADP-linked reactions of the T14A, R46A (*6*), and Q47E enzymes are higher than that of wild-type enzyme, approaching the k_{cat} value for wild-type enzyme in the NAD-linked reaction. Thus, the T14A, R46A, and Q47E mutant enzymes all have weakened $NADP^+$ binding and enhanced catalytic rates for their NADP-linked reactions and display random kinetics when either coenzyme is used. These data reinforce our previous suggestion that a larger portion of the apparent free energy of binding NAD^+ to the enzyme may be used to enhance catalysis than is true for $NADP^+$ binding (6). Removal of those amino acid side chains critical for $NADP^+$ binding (T14A, R46A) or interfering with the role of Arg-46 (Q47E) presumably causes this coenzyme to bind more weakly and with fewer constraints, more like the way NAD^+ normally binds, resulting in catalytic features associated with the NAD-linked reaction.

The enzyme's dual coenzyme specificity and the catalytic mechanisms are influenced not only by the tighter binding of $NADP^+$, governed primarily by Arg-46, but also by the mutual orientations of the coenzyme and G6P during catalysis. We do not know the transition state structures, nor do we have a structure for a ternary complex for the NAD-linked reaction, but our data suggest that Lys-343 and Lys-182, in binding and orienting G6P, play different roles in the NADP- and NAD-linked reactions. On the basis of the fact that both the k_{cat} and coenzyme K_m values for the NADP-linked reaction of the K343Q enzyme are higher than the wild-type enzyme values, typical for the NAD-linked reaction, we investigated this mutant enzyme's kinetic mechanism for the NADP-linked reaction and found that it is random. We interpret this to mean that in binding G6P, Lys-343 helps to orient the substrate so as to allow interaction with NAD^+ but to preclude interaction with $NADP^+$. Without the strong interaction between Lys-343 and the substrate's phosphate moiety, the nonoptimal orientation of G6P relaxes the constraint on the G6P orientation that precludes $NADP^+$ from binding to the enzyme−G6P binary complex, resulting in a random kinetic mechanism. Because of weaker G6P binding to this mutant enzyme, the ordered kinetic pathway for the NADP-linked reaction can only occur at high $NADP^+$ concentration (forcing the enzyme into the $NADP^+$ binary complex) or high G6P concentration (increasing the concentration of G6P bound in an orientation unfavorable for productive $NADP^+$ binding). The nonhyperbolic behavior seen under conditions used to measure true kinetic constants is explained by the availability of two kinetic pathways in the NADP-linked reaction. In the K182Q mutant enzyme, the NAD-linked reaction also shows nonhyperbolic behavior, and in contrast to the results with the K343Q enzyme, both the k_{cat} and coenzyme K_m values are lower than the wild-type values, suggesting characteristics more typical for the NADP-linked reaction. NADH inhibition for this mutant enzyme is noncompetitive with respect to NAD^+, as for the wild-type enzyme. The kinetic constants and nonhyperbolic behavior of the K182Q NAD reaction, as well as the importance of the interaction between Lys-182 and Asp-374 (see below), suggest that Lys-182 plays an important role in facilitating the NAD reaction. Thus, Lys-343 and Arg-46, and perhaps Lys-182, play important roles in the dual coenzyme specificity of *L. mesenteroides* G6PD and help to explain the role of G6P in regulating the enzyme's coenzyme selection.

Roles of Pro-149 and Asp-374. Changing Pro-149 to a Gly or Val residue greatly reduces k_{cat} for both the NADP- and the NAD-linked reactions but also affects all the kinetic constants in both reactions and the dissociation constant for G6P. In P149G G6PD, the Gly residue would permit additional or alternative main chain conformations, some of which might allow the transitions which accompany cis/trans isomerization in the wild-type enzyme. Since Gly only rarely would adopt a cis conformation, the changes are unlikely to be precise. In the P149V enzyme, Val-149 is constrained to be in the trans conformation, so no conformational transition could occur. One possible consequence of the P149V mutation is that it could disfavor the binding of phosphate to the Nϵ2 of His-178 and favor, instead, the Nδ1−phosphate interaction. This might increase the K_d for G6P, which would not be optimally oriented in the active site. The effect of the cis/trans isomerization is particularly important for residues close to the coenzyme domain sheet, for residues in helix α-e in the coenzyme domain, for the conserved peptide, and for residues in the interdomain cleft. The coenzyme site abuts the coenzyme domain sheet and the substrate site is in the interdomain cleft. Isomerization of Pro-149 may play a critical role in enabling the enzyme to adopt the correct transition state conformations. It should be noted, however, that based on the CD spectra, the overall conformation of neither the P149G nor the P149V mutant enzyme is grossly altered.

L. mesenteroides G6PD has an aspartate residue at position 374, and all X-ray structures of this enzyme show that the side chain of this residue interacts with the ζ-amino group of Lys-182 across the interdomain cleft. All other G6PDs (except that found in *L. mesenteroides dextranicus*) contain a glutamine residue in the corresponding position, and in the human enzyme this glutamine interacts with Lys-205, the residue equivalent to Lys-182 (*11*). It is worth noting, also, that both human and *L. mesenteroides* G6PDs can be covalently labeled with pyridoxal 5′-phosphate but that the site of labeling of the human enzyme is Lys-205 (*24*), whereas in the bacterial enzyme Lys-21 and Lys-343 are labeled (*9*). This may result from the interaction of Lys-182 with Asp-374 in *L. mesenteroides* G6PD, rendering it less reactive with pyridoxal 5′-phosphate. An aspartate−lysine interaction is expected to be stronger than the interaction between a glutamine and a lysine residue. This helps, perhaps, to account for the marked stability of the bacterial compared to the human enzyme. The D374Q G6PD is unstable at room temperature, apparently dissociating into its subunits when heated under conditions where the wild-type enzyme remains active. Asp-374 is not located at the dimer interface, but Lys-182 is adjacent to Glu-183, which participates in one of the intersubunit salt bridges (*7*). The

fact that G6P stabilizes the D374Q enzyme much better than either coenzyme and that Lys-182 makes the largest contribution to G6P binding (Table 5) underscores the significance of the interaction between Asp-374 and Lys-182 in G6P binding. For the D374Q enzyme in the NADP-linked reaction, both k_{cat}/K_m values, and in the NADP-linked reaction the substrate k_{cat}/K_m value, are reduced to 15% of their respective wild-type values; the coenzyme k_{cat}/K_m value in the NAD-linked reaction is only 8% of the corresponding wild-type value. This suggests that the interaction between Asp-374 and Lys-182 during catalysis helps to orient Lys-182 optimally for G6P binding, especially in the NAD-linked reaction.

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