Optimizing an Artificial Metabolic Pathway: Engineering the Cofactor Specificity of *Corynebacterium* 2,5-Diketo-D-gluconic Acid Reductase for Use in Vitamin C Biosynthesis†

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ABSTRACT: The strict cofactor specificity of many enzymes can potentially become a liability when these enzymes are to be employed in an artificial metabolic pathway. The preference for NADPH over NADH exhibited by the *Corynebacterium* 2,5-diketo-D-gluconic acid (2,5-DKG) reductase may not be ideal for use in industrial scale vitamin C biosynthesis. We have previously reported making a number of site-directed mutations at five sites located in the cofactor-binding pocket that interact with the 2′-phosphate group of NADPH. These mutations conferred greater activity with NADH upon the *Corynebacterium* 2,5-DKG reductase [Banta, S., Swanson, B. A., Wu, S., Jarnagin, A., and Anderson, S. (2002) *Protein Eng. 15*, 131−140; (*1*)]. The best of these mutations have now been combined to see if further improvements can be obtained. In addition, several chimeric mutants have been produced that contain the same residues as are found in other members of the aldo-keto reductase superfamily that are naturally able to use NADH as a cofactor. The most active mutants obtained in this work were also combined with a previously reported substrate-binding pocket double mutant, F22Y/A272G. Mutant activity was assayed using activity-stained native polyacrylamide gels. Superior mutants were purified and subjected to a simplified kinetic analysis. The simplified kinetic analysis was extended for the most active mutants in order to obtain the kinetic parameters in the full-ordered bi bi rate equation in the absence of products, with both NADH and NADPH as cofactors. The best mutant 2,5-DKG reductase produced in this work was the F22Y/K232G/R238H/A272G quadruple mutant, which exhibits activity with NADH that is more than 2 orders of magnitude higher than that of the wild-type enzyme, and it retains a high level of activity with NADPH. This new 2,5-DKG reductase may be a valuable new catalyst for use in vitamin C biosynthesis.

The ubiquitous intracellular cofactors NAD(H) and NADP(H) differ only by a phosphate group attached to the 2′ position of the adenosine ribose. This difference has been exploited by nature via the evolution of many enzymes that preferentially use one cofactor over the other. The high degree of cofactor specificity of many enzymes is important for proper functioning in natural metabolic pathways, but in a modified or artificial metabolic pathway, this specificity may be undesirable.

An NADPH-dependent 2,5-diketo-D-gluconic acid reductase enzyme has been cloned from *Corynebacterium sp.* (*2*) and has potential commercial value for use in two artificial biosynthetic pathways. The enzyme is able to catalyze the reduction of 2,5-diketo-D-gluconic acid (2,5-DKG)[1] to 2-keto-L-gulonic acid (2-KLG), which is an immediate precursor to L-ascorbic acid, or vitamin C. In a novel engineered in vivo pathway, the enzyme was recombinantly expressed in a 2,5-DKG-producing *Erwinia* strain, resulting in an organism that could produce 2-KLG from glucose in a single fermentation step (*2*−*4*). Moreover, in a recently developed in vitro reaction scheme, the reductase enzyme was added to a bioreactor containing stationary *Erwinia* cells deficient in their native glucose dehydrogenase plus a purified $NADP^+$-dependent glucose dehydrogenase. The two free enzymes were able to recycle the cofactor outside of the cells, resulting in in vitro conversion of glucose to 2-KLG (*5*).

Both of these processes have been shown to work using an NADPH-dependent 2,5-DKG reductase. However, NADH is less costly, more prevalent in the cell, and more stable than NADPH, and, therefore, process improvements could

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[1] Abbreviations: 2,5-DKG, 2,5-diketo-D-gluconic acid; 2-KLG, 2-keto-L-gulonic acid; AKR, aldo-keto reductase; R3αHSD, rat liver 3α-hydroxysteroid dehydrogenase; PtXR, *Pachysolen tannophilus* xylose reductase; Sl3D3βR, *Spodoptera littoralis* 3-dehydroecdysone 3β-reductase.

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be obtained in both of these engineered metabolic pathways if the natural cofactor specificity of the enzyme could be broadened to enable the use of NADH. For that reason, we are attempting to improve, using rational protein design, the activity of the *Corynebacterium* 2,5-DKG reductase with NADH as a cofactor.

Considerable information already exists in the literature on the engineering of the cofactor specificity of other enzymes. The first example of engineering cofactor specificity was described by Scrutton et al. with the enzyme glutathione reductase (*6*). This protein contains a "Rossman"-type dinucleotide-binding fold, and a number of further studies on similar proteins have exemplified how to alter the cofactor specificity of enzymes with this motif (*6*). The 2,5-DKG reductase enzyme does not contain the "Rossman" fold. It is a member of the aldo-keto reductase (AKR) superfamily of proteins, which is characterized by the existence of the bound cofactor in a unique extended conformation (*7*). Mutations affecting cofactor binding have been made in other members of the AKR superfamily (*8*−*12*). However, as far as we are aware, no other members of the AKR superfamily have been engineering for improved enzyme activity with nonpreferred cofactors.

The 2,5-DKG reductase is a 34 kDa monomer (*13*), and the structure of the enzyme with a bound NADPH molecule has been solved at 2.1 Å resolution, which allows us to see the details of interactions between the enzyme and the cofactor. Like other members of the AKR superfamily, 2,5-DKG reductase appears to employ the ordered bi bi kinetic mechanism with cofactor binding first and leaving last (*14*).

Previous site-directed mutagenesis work with this enzyme led to improvements in catalysis. Mutations made in the substrate-binding pocket of the enzyme resulted in the double mutant F22Y/A272G, which appeared to significantly improve the V_{max} of the enzyme when NADPH was used as a cofactor (*15*). Another, evolutionarily related *Corynebacterium* enzyme, 2,5-DKG reductase B, with distinct kinetic parameters, has been reported, but it also has a preference for NADPH (*16*).

Most members of the AKR superfamily appear to naturally have a strong preference for NADPH over NADH (*12*). However, there are a few members of the family that are able to use both cofactors. For example, the 3α-hydroxysteroid dehydrogenase from rat liver (R3αHSD), which is a well-studied member of the AKR superfamily, has been shown to have apparent K_Ms of 1.9 and 39.8 μM for NADPH and NADH, respectively (*11*). In the yeast *Pachysolen tannophilus*, a xylose reductase (PtXR) has been cloned that is thought to have dual cofactor specificity (*17*). A 3-dehydroecdysone 3β-reductase (Sl3D3βR) has also been cloned from *Spodoptera littoralis*, or the cotton leafworm (*18*). It has been reported to have an apparent K_M of 0.94 μM for NADPH and 22.8 μM when NADH is used as a cofactor (*19*). As far as we are aware, there are no members of the family that are naturally specific for NADH only.

We have previously reported a study in which site-directed point mutations were made at all of the amino acid positions in the cofactor-binding site that exhibit a direct interaction with the 2′-phosphate of NADPH in order to explore cofactor specificity (Figure 1) (*1*). The mutations were made at Lys232, Ser233, Val234, Arg235, and Arg238, and it was discovered that mutations at Lys232, Arg235, or Arg238 could lead to improvements in catalysis with NADH. Using a native gel assay and kinetic analyses, we were able to identify the following single mutants as having improved activity with NADH: K232G, R235G, R235T, R238E, and R238H. The best single mutant obtained, R238H, produced an almost 7-fold improvement in catalysis with NADH over the wild type. This improvement appeared to be mainly due to the stabilization of the transition-state, and not due to a major increase in the ground-state binding affinity for NADH (*1*).

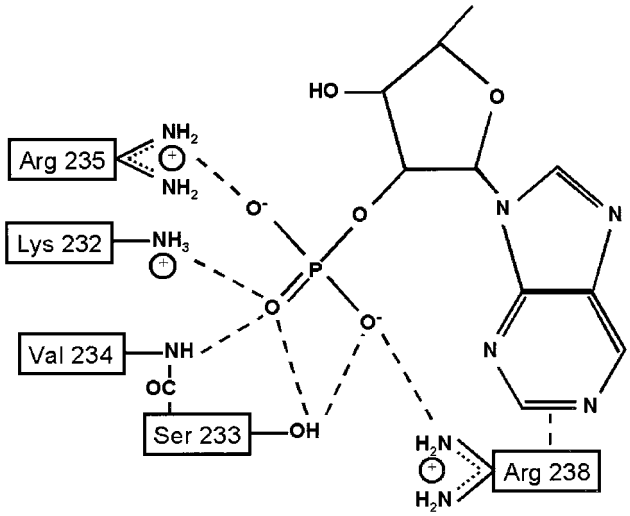

FIGURE 1: Amino acids in the wild-type 2,5-DKG reductase A that make noncovalent interactions with the 2′-phosphate group of NADPH. Only the adenosine moiety of the NADPH molecule is shown. Arg238 lies flush against the adenine ring in an apparent stacking interaction. The role of Arg235 in the cofactor-binding pocket has not previously been discussed in the literature.

In this paper, we report the results of combining the best single mutations to make all possible double and triple mutations at these three cofactor-interacting residues. In addition, we have made chimeric mutants where the residues that interact with the 2′-phosphate of NADPH were substituted with the corresponding residues in other members of the AKR superfamily that naturally exhibit some activity with NADH. Finally, the best mutations obtained by these methods were combined with the F22Y/A272G double mutant to see if further improvements in the kinetic parameters could be obtained when NADH was used as a cofactor.

The best mutants produced have been purified to homogeneity, and their kinetic parameters have been measured in the forward direction with both NADPH and NADH as cofactors. The F22Y/K232G/R238H/A272G quadruple mutant exhibited the greatest improvement, with a 110-fold increase in activity with NADH compared to that of the wild-type enzyme, and only a minor reduction in activity with NADPH. These improvements may eventually prove to be attractive for use in industrial-scale vitamin C biosynthesis.

MATERIALS AND METHODS

Materials. Oligonucleotide primers were from either Life Technologies or Integrated DNA Technologies. AG 50W-X2 cation-exchange resin and bromophenol blue were from Bio-Rad. MOPS, Bis-Tris, sodium gluconate, aniline, phthalic acid, NADH, and NADPH were from Sigma.

1-Butanol was from Fisher. Restriction enzymes were from NEB. Ligase was from NEB or Roche. Pre-cast polyacrylamide gradient gels were from Novex. Precoated cellulose TLC plates were from Macherey-Nagel. All other materials are the same as previously described (*1*).

Site-Directed Mutagenesis. Cassette mutagenesis was performed using the pATP003.xb vector, which contains a GroES fragment fused to the amino terminus of the 2,5-DKG reductase gene, and promotes high levels of expression in *E. coli* (*20*). This plasmid has been previously engineered to contain a silent, unique *Bsu*36I restriction site upstream, and a silent, unique *Xho*I restriction site downstream of the region to be mutated. Mutations were prepared as previously described (*1*). The codons used for the double and triple mutants are as follows: K232G (GGC), K232S (AGC), R235G (GGC), R235T (ACG), R235E (GAA or GAG), and R238H (CAC). Every possible double and triple mutant was made using these single mutations. The use of "wild card" bases allowed for several mutants to be obtained in a single mutagenic cassette.

For the chimeric mutants, the following codons were used: R3αHSD: K232R (CGC), V234F (TTC), and R235N (AAC); Sl3D3βR: S233T (ACG) and R235S (TCG); PtXR: V234T (ACG), R235F (TTC), and R238T (ACG). For the R3αHSD and Sl3D3βR oligonucleotides, the codon at R238 was chosen to be (CRC) so that both arginine and histidine side chains would be obtained. This was not possible with the PtXR mutant where a threonine was required at the 238 position.

Combining the F22Y/A272G Double Mutant. The F22Y/A272G double mutant was previously made in the ptrp1−35 vector (*15*). These two mutations were subcloned into the ptrp1−35.xb vector, which contained the unique cassette mutagenesis restriction sites. The F22Y mutation was transferred using a *Mlu*I/*Xba*I double digestion, and the A272G mutation was transferred using a *Bam*HI/*Kpn*I double digestion. The new construct was then moved into the final vector, pATP003.xb, with an *Eco*RI/*Hin*dIII double digestion. The cassettes from the best mutants obtained were subcloned into the F22Y/A272G double mutant construct using the unique *Bsu*36I and *Xho*I restriction sites. All of the mutants were sequenced for accuracy.

Activity-Stained Native Gels. Mutant plasmids were transformed into JM109 *E. coli* cells, and the new proteins were induced and expressed in 50 mL cultures as previously described (*1*). The crude protein lysates containing each of the mutants were loaded onto native polyacrylamide gels in order to assay their cofactor specificities. Identical gels were soaked in 1 mM solutions of each cofactor and overlaid with filter paper soaked in 20 mM 2,5-DKG as described by Seymour and Lazarus (*21*). Gels were observed under UV light to determine the existence of activity with either cofactor.

Protein Expression and Purification. The most interesting mutants on the native gels were purified to homogeneity as previously described for use in kinetic analysis (*1*). The concentration of the proteins was determined by measuring their absorbance at 280 nm and using an extinction coefficient of 43 430 M^{-1} cm^{-1} for the double, triple, and chimeric mutants. An extinction coefficient of 44 920 M^{-1} cm^{-1} was used for mutants containing the F22Y mutation (*22*). The protein concentrations were verified by the method of Bradford (*23*), and the homogeneity was observed using SDS−PAGE under reducing conditions (*24*).

Substrate Preparation. 2,5-DKG was prepared from sodium gluconate using stationary *P. citrea* cells (ATCC 39140) as previously described (*1*). To improve the quality of the substrate for this work, the 2,5-DKG was purified away from the unconverted gluconate and any intermediates. The 2,5-DKG-containing product was loaded onto a column containing AG 50W-X2 cation-exchange resin using an FPLC system (*13*). The free acid form of 2,5-DKG was eluted from the column with water, and active fractions were pooled together. The elutant was then buffered with 50 mM MOPS, and the pH was raised to 6.5 with NaOH.

The purity of the solution was assayed using thin-layer chromatography. A mixture of 1-butanol−acetic acid−water was used to separate the carbohydrate solution (*25*). Identical chromatograms were developed with either bromophenol blue or aniline hydrogen phthalate reagent (*26*) to monitor the presence of gluconate and 2,5-DKG. The concentration of the final 2,5-DKG product was determined enzymatically, as previously described (*1*).

Enzyme Kinetics. Kinetic reactions were performed in 96 well microtiter plates and read with a temperature-controlled SpectraMax 190 plate reader (Molecular Devices). All reactions were performed in a volume of 200 μL, at 25 °C, buffered with 100 mM Bis-Tris, pH 7.0. The initial rates of oxidation of NADH were followed at 375 nm, and the initial rates of oxidation of NADPH were followed at 340 nm. The absorbance at 375 nm was used to record the NADH data because large concentrations or NADH were required to saturate the enzyme, and the absorbance at 340 nm resulted in readings that were outside of the linear range of the spectrophotometer. Both substrate and cofactor concentrations were varied, and the data were fit to the kinetic rate equations found under Results. At least three different substrate concentrations were used. Measurements were done in at least triplicate and averaged, and the nonlinear regression of the unweighted data was performed with the NLREG program (*27*).

RESULTS

Double, Ttriple, and Chimeric Mutant Construction. Oligonucleotide cassettes with "wild card" bases were used to construct the following double and triple mutants by combining the most successful single mutants: K232G/R235G, K232G/R235T, K232G/R238E, K232G/R238H, R235G/R238E, R235G/R238H, R235T/R238E, R235T/R238H, K232G/R235G/R238E, K232G/R235T/R238E, K232G/R235G/R238H, and K232G/R235T/R238H. In addition, the single mutant with the poorest kinetic parameters with NADH as a cofactor, K232S (*1*), was used to make double and triple mutants to further explore the effects of mutational additivity. These mutants were K232S/R235G, K232S/R235T, K232S/R238E, K232S/R238H, K232S/R235G/R238E, K232S/R235T/R238E, K232S/R235G/R238H, and K232S/R235T/R238H. Finally, chimeric mutants were constructed (Table 1), and the R238H mutation was added with a "wild card" base whenever possible. These mutants were K232R/V234F/R235N, K232R/V234F/R235N/R238H, S233T/R235S, S233T/R235S/R238H, and V234T/R235F/R238T.

Table 1: Sequence Alignments of the 2′-Phosphate-Binding Residues for Members of the AKR Superfamily That Can Use NADH as a Cofactor, Aligned with the *Corynebacterium* 2,5-DKG Reductase A Sequence

	232	233	234	235	238
2,5-DKGRA[a]	K	S	V	R	R
R3αHSD[b]	R	S	F	N	R
PtXR[c]	K	S	T	F	T
Sl3D3βR[d]	K	T	V	S	R

[a] Corynebacterium 2,5-DKG reductase A. [b] Rat liver 3α-hydroxysteroid dehydrogenase. [c] *Pachysolen tannophilus* xylose reductase. [d] *Spodoptera littoralis* 3-dehydroecdysone 3β-reductase.

Table 2: Native Gel Results for Double, Triple, and Chimeric Mutants[a]

	relative expression level	NADPH activity	NADH activity
wild type	**	***	
K232G/R235G	***	*	*
K232G/R235T	***	*	
K232G/R238E	***	**	
K232G/R238H	***	***	***
K232S/R235G	***		
K232S/R235T	***		
K232S/R238E	***		
K232S/R238H	***	***	**
R235G/R238E	***		
R235G/R238H	***	***	*
R235T/R238E	***		
R235T/R238H	***	***	*
K232G/R235G/R238E	***	**	
K232G/R235T/R238E	***		
K232G/R235G/R238H	***	**	***
K232G/R235T/R238H	***	***	***
K232S/R235G/R238E	***		
K232S/R235T/R238E	***		
K232S/R235G/R238H	***		
K232S/R235T/R238H	***	*	
K232R/V234F/R235N	***		
K232R/V234F/R235N/R238H	***	*	
S233T/R235S	***	***	*
S233T/R235S/R238H	***	***	**
V234T/R235F/R238T	*		

[a] Identical native polyacylamide gels were soaked in either NADH or NADPH and overlaid with filter paper containing the substrate. Gels were observed under UV light to see where cofactor had been oxidized. Gels were then Coomassie stained to visualize the relative protein content in each band. The activity with each cofactor was not corrected for the variation in expression levels.

Activity-Stained Native Gel Assay Results. The mutants were expressed in *E. coli* JM109 cells, and the crude protein lysates were run on native polyacrylamide gels and activity-stained (Table 2). By running identical gels with comparable expression levels and using either NADH or NADPH as cofactors, this assay allowed for the rapid qualitative determination of the cofactor specificity of the new mutants without any purification (*21*).

Several trends observed with the single mutants were retained when the double and triple mutants were combined and examined using the native gel assay. At the Lys232 position, the single mutation to glycine demonstrated improved activity with NADH, while the single mutation to serine showed a reduction in activity (*1*). This trend was generally retained in all of the double and triple mutants, except for the surprising K232S/R238H double mutant. This mutant appeared to be more active than the R238H mutant alone on the NADH-stained gel (*1*), which suggests that the effects of these mutations are not always additive.

At the 238 position, the R238E single mutation has previously been shown to result in an increase in activity with NADH, but this mutation eliminated detectable activity with NADPH (*1*). This effect was retained whenever R238E was combined into a double or triple mutant. These proteins represent true cofactor specificity reversal mutants by improving activity with NADH and eliminating activity with NADPH. As previously reported (*1*), a histidine at the 238 position was the best single mutant obtained, and it also produced superior double and triple mutants. From the native gel results, the best enzymes obtained appeared to be K232G/R238H, K232G/R235G/R238H, and K232G/R235T/R238H.

Mutations at the Arg235 position did not seem to have a significant impact on cofactor specificity based on the native gel results. For example, the double mutants R235G/R238H and R235T/R238H appeared to be similar to the R238H mutant alone. The three best mutants, K232G/R238H, K232G/R235G/R238H, and K232G/R235T/R238H, appear to be similar on the native gels, which suggests that the combination of the K232G and R238H mutations is primarily responsible for the improvement in activity and the effects of mutations at Arg235 are minor.

The chimeric mutants (Tables 1 and 2) displayed more variable behavior on the native gels. The PtXR mutant appeared to suffer from a low expression level of protein, which suggests that these mutations have a detrimental affect on the overall stability of the protein. The R3αHSD mutants produced stable proteins, but the mutations appeared to have deleterious effects on the cofactor-binding pocket, as evidenced by losses in activity with NADPH and no apparent improvement in activity with NADH. The only chimeric mutant cofactor-binding pocket substitutions that appeared to improve activity with NADH were the S233T/R235S double mutant (Sl3D3βR) and the triple mutant made by adding the R238H mutation. The S233T and R235S single mutations have both been examined previously, and no increase in activity with NADH was detected with either one separately (*1*); however, with the two mutations combined, a definite increase in activity with NADH was seen on the native gel assay. As expected, the effects were enhanced by the addition of the R238H mutation. These mutants were also active with NADPH as a cofactor.

Simplified Steady-State Kinetics. The most interesting double, triple, and chimeric mutants were purified to homogeneity, using methods previously described (*1*). These mutants were then subjected to a simplified kinetic analysis to directly compare some of their respective kinetic parameters. The rate equation for the ordered bi bi mechanism in the forward direction in the absence of products (eq 1) contains four adjustable parameters:

$$\frac{dP}{dt} = \frac{E_t k_{cat} AB}{K_{ia}K_b + K_b A + K_a B + AB} \quad (1)$$

where dP/dt is the rate of product formation, E_t is the total enzyme concentration, k_{cat} is the turnover number, A is the concentration of cofactor, B is the concentration of substrate, K_{ia} is the dissociation constant for enzyme−cofactor complex, K_a is the Michaelis constant for cofactor, and K_b is the Michaelis constant for substrate (*28*).

Table 3: Kinetic Parameters with NADH as a Cofactor Using the Modified Rate Equation[a]

	k_{cat}/K_b ($min^{-1}\,mM^{-1}$)	K_{ia} (mM)	k_{cat}/K_bK_{ia} ($min^{-1}\,mM^{-2}$)	$(k_{cat}/K_b)/(k_{cat}/K_b)_{wt}$	$(K_{ia})/(K_{ia})_{wt}$	$(k_{cat}/K_bK_{ia})/(k_{cat}/K_bK_{ia})_{wt}$
wild type	1.0 ± 0.1	2.2 ± 0.2	0.45 ± 0.06	1.0	1.0	1.0
wild type[b]	0.95 ± 0.04	2.6 ± 0.2	0.37 ± 0.03	0.95	1.2	0.8
R238H[b]	5.1 ± 0.2	2.1 ± 0.1	2.4 ± 0.1	5.1	0.95	5.3
F22Y/A272G	1.7 ± 0.2	2.1 ± 0.4	0.81 ± 0.20	1.7	1.0	1.8
K232G/R238E	7.3 ± 1.2	3.5 ± 0.8	2.1 ± 0.6	7.3	1.6	4.6
K232G/R238H	9.1 ± 0.4	0.73 ± 0.08	12 ± 1	9.1	0.33	27
K232S/R238H	3.3 ± 0.2	1.1 ± 0.1	3.0 ± 0.3	3.3	0.50	6.6
K232G/R235G/R238E	12 ± 1	3.0 ± 0.2	4.0 ± 0.4	12	1.4	8.8
K232G/R235G/R238H	8.0 ± 0.4	2.4 ± 0.2	3.3 ± 0.3	8.0	1.1	7.3
K232G/R235T/R238H	8.4 ± 0.2	1.7 ± 0.1	4.9 ± 0.3	8.4	0.77	11
S233T/R235S/R238H	8.6 ± 0.2	2.4 ± 0.1	3.6 ± 0.2	8.6	1.1	7.9
F22Y/K232G/R238H/A272G	24 ± 1	0.82 ± 0.09	29 ± 3	24	0.37	64
F22Y/K232G/R235G/R238E/A272G	27 ± 4	3.2 ± 0.7	8.4 ± 2.2	27	1.5	19
F22Y/K232G/R235G/R238H/A272G	20 ± 1	3.6 ± 0.3	5.6 ± 0.5	20	1.6	12
F22Y/K232G/R235T/R238H/A272G	25 ± 1	2.4 ± 0.2	10 ± 1	25	1.1	23
F22Y/S233T/R235S/R238H/A272G	15 ± 2	3.1 ± 0.6	4.8 ± 1.1	15	1.4	11

[a] The rate equation can be found as eq 2 under Results. The $k_{cat}B/K_b$ term becomes the apparent k_{cat}. The K_{ia} term becomes the apparent K_a. The term $k_{cat}B/K_bK_{ia}$ is then the apparent k_{cat}/K_a. All reactions were performed in triplicate in 100 mM Bis-Tris, pH 7.0, at 25 °C. [b] These data was previously obtained with unpurified 2,5-DKG and 50 mM Bis-Tris (*1*).

This equation can be simplified if the concentrations of 2,5-DKG used are below the K_M for substrate. The equation then reduces to have only two adjustable parameters (eq 2), and is useful for comparing the cofactor-dependent activity of different mutants, without having to collect data points that saturate the enzyme with the substrate, 2,5-DKG (*1*). The results from this preliminary kinetic characterization can be seen in Table 3. In this equation, $k_{cat}B/K_b$ becomes the apparent k_{cat}, and K_{ia} becomes the apparent K_a. Since the apparent k_{cat} contains the substrate concentration, B, the constant parameter k_{cat}/K_b will be used for comparison.

$$\frac{dP}{dt} = \frac{E_t\left(\frac{k_{cat}}{K_b}\right)AB}{K_{ia} + A} \qquad (2)$$

This kinetic characterization was previously used to compare the activity of the single mutants. Table 3 also contains the wild-type and R238H data from the previous single mutant work, which was done with unpurified substrate and 50 mM Bis-Tris as a buffer (*1*). The wild-type results were found to be very similar using both sets of conditions.

In the previous work with the single mutants, it was observed that when improvements in NADH-mediated activity were obtained, it was due to an increase in the apparent k_{cat}, or k_{cat}/K_b, term, and not due to a large improvement in the apparent K_M, or K_{ia}, term (*1*). Almost all of the double and triple mutants further extended the improvements in the k_{cat}/K_b term, with the K232G/R235G/R238E mutant exhibiting the largest increase in k_{cat}/K_b of 12-fold over the wild type.

In contrast to the results with the single mutants, three of the new mutants exhibited significant decreases in the K_{ia} term. The K232G/R238H mutant exhibited the greatest decrease, from 2.2 to 0.73 mM. The K_{ia} term is the dissociation constant for the cofactor. The only mutants that resulted in a significant increase in the K_{ia} term over the wild type were the enzymes containing the R238E mutation.

The ratio of $k_{cat}/K_{ia}K_b$ is a useful parameter to compare the total improvements made to the mutant enzymes. These ranged from a modest 4.6-fold improvement over the wild type with the K232G/R238E double mutant to a 27-fold improvement with the best mutant, K232G/R238H.

Table 4: Native Gel Results for F22Y/A272G-Containing Mutants[a]

	relative expression level	NADPH activity	NADH activity
wild type	**	***	
F22Y/A272G	**	***	
F22Y/K232G/R238E/A272G	***		**
F22Y/K232G/R238H/A272G	***	***	***
F22Y/K232G/R235G/R238E/A272G	***		**
F22Y/K232G/R235G/R238H/A272G	***	*	**
F22Y/K232G/R235T/R238H/A272G	***	**	**
F22Y/S233T/R235S/R238H/A272G	**	***	*

[a] Native gels results were obtained as described in Table 2.

F22Y/A272G Mutants. A subset of the mutants discussed above was combined with the previously obtained F22Y/A272G double mutant (*15*). This mutant demonstrates superior activity with NADPH compared to the wild-type enzyme, and it was hoped that these improvements would be additive with respect to the cofactor-binding pocket mutants made in this work. Therefore, the following mutants were combined with the F22Y/A272G double mutant: K232G/R238H, K232G/R235G/R238E, K232G/R235G/R238H, K232G/R235T/R238H, and S233T/R235S/R238H. The mutant proteins were run out on native gels and analyzed qualitatively as before (Table 4).

The F22Y/A272G mutants were also purified and subjected to the simplified kinetic analysis using eq 2 (Table 3). The addition of the F22Y/A272G double mutations appeared to dramatically improve the k_{cat}/K_b term while concomitantly slightly increasing the K_{ia} term. For almost every cofactor specificity mutant, the value of k_{cat}/K_bK_{ia} approximately doubled when these were combined with F22Y/A272G. Such consistent additivity is not surprising since the F22Y and A272G mutation sites are distal to the cofactor specificity site (*15*). The best mutant obtained was the F22Y/K232G/R238H/A272G quadruple mutant with a k_{cat}/K_bK_{ia} value that was 64-fold improved over the wild-type enzyme.

Table 5: Changes in the Ground- and Transition-State Binding Energies[a]

	$\Delta\Delta G_b$ (kcal/mol)	$\Delta\Delta G_b^{\ddagger}$ (kcal/mol)	sum of $\Delta\Delta G_b^{\ddagger}$ from individual mutants	sum of $\Delta\Delta G_b^{\ddagger}$ by adding F22Y/A272G
K232G[b]	−0.16	−0.29		
K232S[b]	0.044	0.53		
R235G[b]	0.69	−0.25		
R235T[b]	0.72	0.34		
R238E[b]	0.69	0.25		
R238H[b]	−0.13	−1.1		
K232G/R238E	0.27	−0.90	−0.04	
K232G/R238H	−0.65	−2.0	−1.4	
K232S/R238H	−0.41	−1.1	−0.59	
K232G/R235G/R238E	0.18	−1.3	−0.29	
K232G/R235G/R238H	0.05	−1.2	−1.7	
K232G/R235T/R238H	−0.15	−1.4	−1.1	
S233T/R235S/R238H	0.05	−1.2		
F22Y/A272G	−0.03	−0.34		
F22Y/K232G/R238H/A272G	−0.58	−2.5		−2.3
F22Y/K232G/R235G/R238E/A272G	0.22	−1.7		−1.6
F22Y/K232G/R235G/R238H/A272G	0.29	−1.5		−1.5
F22Y/K232G/R235T/R238H/A272G	0.052	−1.9		−1.8
F22Y/S233T/R235S/R238H/A272G	0.20	−1.4		−1.6

[a] The contribution to the ground-state binding energy is given by: $\Delta\Delta G_b = -RT \ln[(K_{ia})_{wt}/(K_{ia})_{mut}]$, and the contribution to the transition-state binding energy is given by: $\Delta\Delta G_b^{\ddagger} = RT \ln[(k_{cat}/K_{ia}K_b)_{wt}/(k_{cat}/K_{ia}K_b)_{mut}]$. [b] From data previously reported (*1*).

Cofactor Binding Energies. The parameters obtained using the simplified rate equation can be used to estimate the changes in both ground-state and transition-state binding energies. The change in ground-state binding energy can be obtained from the ratio of the dissociation constants for the cofactor as compared to the wild type (eq 3) where R is the gas constant and T is the temperature in degrees kelvin (*29*).

$$\Delta\Delta G_b = (\Delta G_b)_{mut} - (\Delta G_b)_{wt} = -RT \ln[(K_{ia})_{wt}/(K_{ia})_{mut}] \quad (3)$$

The dissociation constant, K_{ia}, is the apparent K_M in eq 2. The changes in the ground-state binding energies can be found in Table 5.

The changes in ground-state binding energy were minor, with the biggest changes seen in the K232G/R238H mutant alone and when it was combined with the F22Y/A272G double mutant. These mutants gained 0.65 and 0.58 kcal/mol of binding energy, respectively. The K232S/R238H double mutant also surprisingly gained 0.41 kcal/mol.

The ratio of k_{cat}/K_a can be used to calculate the change in the transition-state binding energies (*29*). From the simplified rate equation, the term $k_{cat}B/K_{ia}K_b$ becomes the apparent k_{cat}/K_a. Therefore, the change in transition-state binding energy can be estimated using eq 4. The results for each mutant can also be found in Table 5.

$$\Delta\Delta G_b^{\ddagger} = RT \ln\left[\left(\frac{k_{cat}}{K_{ia}K_b}\right)_{wt} / \left(\frac{k_{cat}}{K_{ia}K_b}\right)_{mut}\right] \quad (4)$$

There were significant increases in the transition-state binding energy with every new mutant, including a gain of 2.0 kcal/mol for the K232G/R238H double mutant and the largest gain of 2.5 kcal/mol for the F22Y/K232G/R238H/A272G quadruple mutant.

The values of the changes in transition-state binding energy can be used to see if the combination of mutations produces additive effects. The free energies previously obtained with the single mutations can be added together and compared to the free energy change obtained when the mutations were combined together. The same comparison can be made between the double and triple mutants before and after they were combined with the F22Y/A272G double mutant (Table 5).

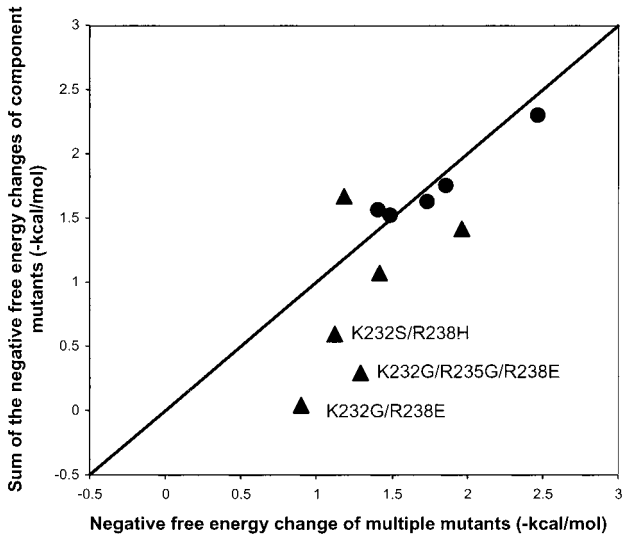

FIGURE 2: Comparison of the additivity of the transition-state free energies of different cofactor specificity mutants using NADH as a cofactor. The horizontal axis contains the free energy change of the multiple mutants, and the vertical axis contains the sum of the free energies of the component mutations. Points along the diagonal line demonstrate perfect free energy additivity. The combination of the single mutants is shown as (▲). The combination of the double and triple mutants (treated as single components) with the F22Y/A272G mutations is shown as (●). The three mutants that are most distant from the diagonal, and thus benefit from the largest gains in nonadditive transition-state binding energy, are labeled.

The values of the sums of the negative free energy changes and the actual negative free energy changes can be plotted for comparison (Figure 2). Points displaced from the diagonal indicate a lack of additivity. When the single mutants were combined to form double and triple mutants, there was a large degree of scatter, indicating that the transition-state free

Table 6: Full Kinetic Parameters with NADH as a Cofactor[a]

	k_{cat} (min^{-1})	K_{ia} (mM)	K_a (mM)	K_b (mM)
wild type	170 ± 70	2.4 ± 1.5	1.4 ± 1.3	130 ± 68
F22Y/A272G	74 ± 22	1.8 ± 1.0	2.4 ± 1.2	30 ± 16
K232G/R238H	1200 ± 800	0.81 ± 0.78	0.66 ± 1.2	110 ± 89
F22Y/K232G/R238H/A272G	990 ± 240	0.66 ± 0.17	1.2 ± 0.3	32 ± 7
F22Y/K232G/R235G/R238E/A272G	600 ± 220	4.2 ± 3.1	3.4 ± 1.7	13 ± 9
F22Y/K232G/R235G/R238H/A272G	1100 ± 400	3.5 ± 0.7	2.7 ± 0.8	43 ± 14
F22Y/K232G/R235T/R238H/A272G	4100 ± 3300	1.9 ± 0.5	5.2 ± 4.4	150 ± 120
F22Y/S233T/R235S/R238H/A272G	510 ± 280	3.1 ± 0.8	3.9 ± 2.0	27 ± 14

[a] All reactions were performed in triplicate in 100 mM Bis-Tris, pH 7.0, at 25 °C.

energy changes are not additive. The fact that most of the scattered points were below the diagonal suggests that those mutants benefit from a gain in transition-state binding energy that is greater than the sum of the individual component mutations. As previously mentioned, the K232S/R238H double mutant clearly benefits from nonadditive improvements, but the two data points that are the most distant from the diagonal, and thus obtain the greatest increase in nonadditive transition-state binding energy, are the K232G/R235G/R238E and K232G/R238E mutants.

When the double and triple mutants were combined with the F22Y/A272G double mutant, the points all were close to the diagonal. The transition-state free energy changes gained by adding the F22Y/A272G double mutant were additive in every case. This is consistent with cofactor specificity mutants being close enough to interact with each other [$\Delta\Delta G_I \neq 0$; (*30*)] but far enough away from the other two residues (22 and 272) to be essentially independent of changes at these sites.

Extended NADH Kinetics. The kinetic data sets obtained for the best mutants were extended using higher 2,5-DKG concentrations, so that the parameters in the full rate equation in the forward direction (eq 1) could be estimated (Table 6). A comparison of the k_{cat}/K_b and K_{ia} values obtained using the full rate equation and the parameters obtained in the kinetic simplification (Table 3) shows good agreement, which validates the use of the simplified equation for comparing the cofactor-dependency of the different mutants.

The highest k_{cat} value was obtained with the F22Y/K232G/R235T/R238H/A272G mutant with a value of 4100 min^{-1}. The lowest K_a value of 0.66 mM was seen with the K232G/R238H mutant, and the lowest K_{ia} value of 0.66 mM was obtained with the F22Y/K232G/R238H/A272G quadruple mutant. The lowest K_b value, 13 mM, was found with the F22Y/K232G/R235G/R238E/A272G quintuple mutant.

The full kinetic parameters can also be used to compare the effects of individual mutations. A comparison of the wild type and F22Y/A272G mutants show that the addition of the F22Y/A272G mutations results in a decrease in k_{cat}, K_{ia}, and K_b, while an increase in K_a is observed. This trend is identical to what is seen when the F22Y/A272G mutations are added to the K232G/R238H double mutant.

The F22Y/K232G/R235G/R238E/A272G mutant and the F22Y/K232G/R235G/R238H/A272G mutant only differ at the 238 position, where either a glutamic acid or a histidine side chain is found. This small change produces dramatic consequences. As mentioned previously, the glutamic acid residue eliminates detectable activity with NADPH. As compared to the histidine-containing mutant, it produced a deleterious effect in every kinetic parameter, except the K_b term, which was lowered to 13 mM (the best value obtained in this work). This suggests that this charge reversal mutation results in a change in the conformation of the cofactor-binding pocket of the enzyme. This different conformation exhibits a poorer affinity for the NADH cofactor, as seen by the largest K_{ia}, or dissociation constant obtained in this work. However, once the cofactor is bound, it results in improvements in the geometry of the substrate-binding pocket, as evidenced by a k_{cat}/K_b value that is twice as large as is found with the histidine-containing mutant.

The three mutants F22Y/K232G/R238H/A272G, F22Y/K232G/R235G/R238H/A272G, and F22Y/K232G/R235T/R238H/A272G only differ at the 235 position, where glycine, threonine, or the native arginine is found. The mutants containing arginine or glycine have similar values of k_{cat} and K_b. The threonine-containing mutant exhibits a significant increase in both values, but as they are proportional, all three mutants have a similar k_{cat}/K_b value. This suggests that mutations of the side chain at the 235 position do not have a large effect on the conformation of the substrate-binding pocket, and thus do not affect the turnover of the enzyme once the cofactor has bound. However, mutations at this residue do affect the affinity of the enzyme for NADH, as seen by the changes in K_{ia} and K_a. The glycine-containing mutant exhibits the highest K_{ia} of 3.5 mM, and the threonine-containing mutant has the highest K_a of 5.2 mM. The original arginine side chain results in a K_{ia} of 0.66 and a K_a of 1.2. Although mutations at the 235 position resulted in single mutants with improved NADH activity, it now appears that the native arginine can form interactions that favor NADH when combined with other mutations.

For the enzymatic reaction in the absence of products, the first two mechanistic steps are as follows, where E is the free enzyme, A is the cofactor, B is the substrate, EA is the enzyme−cofactor complex, and EAB is the ternary complex of enzyme, cofactor, and substrate:

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\leftrightarrow}} \mathrm{EA}$$

$$\mathrm{EA} + \mathrm{B} \overset{k_3}{\Rightarrow} \mathrm{EAB} \qquad (5)$$

These rate constants can be obtained from the following relationships: $k_1 = k_{cat}/K_a$, $k_2 = k_{cat}K_{ia}/K_a$, and $k_3 = k_{cat}/K_b$ (Table 7) (*28*). The F22Y/K232G/R238H/A272G quadruple mutant appears to be the best overall enzyme when NADH is used as a cofactor, with the lowest K_{ia} value (k_2/k_1) and the highest value of k_3. Comparing the ratios of k_1k_3/k_2 shows that the quadruple mutant is improved 110-fold over the wild-type enzyme (Table 7, Figure 3).

Table 7: Comparison of Individual Rate Constants with NADH as a Cofactor[a]

	k_1 (mM min^{-1})	k_2 (min^{-1})	k_3 (mM min^{-1})	k_1k_3/k_2 (min^{-1})	$(k_1k_3/k_2)/(k_1k_3/k_2)_{wt}$
wild type	1.3	3.1	1.0	0.42	1.0
F22Y/A272G	22	40	2.6	1.4	3.4
K232G/R238H	1800	1500	11	14	32
F22Y/K232G/R238H/A272G	830	550	31	47	110
F22Y/K232G/R235G/R238E/A272G	180	760	43	10	24
F22Y/K232G/R235G/R238H/A272G	410	1400	26	7.4	18
F22Y/K232G/R235T/R238H/A272G	790	1500	27	14	34
F22Y/S233T/R235S/R238H/A272G	130	400	19	6.1	15

[a] $k_1 = k_{cat}/K_a$, $k_2 = k_{cat}K_{ia}/K_a$, $k_3 = k_{cat}/K_b$.

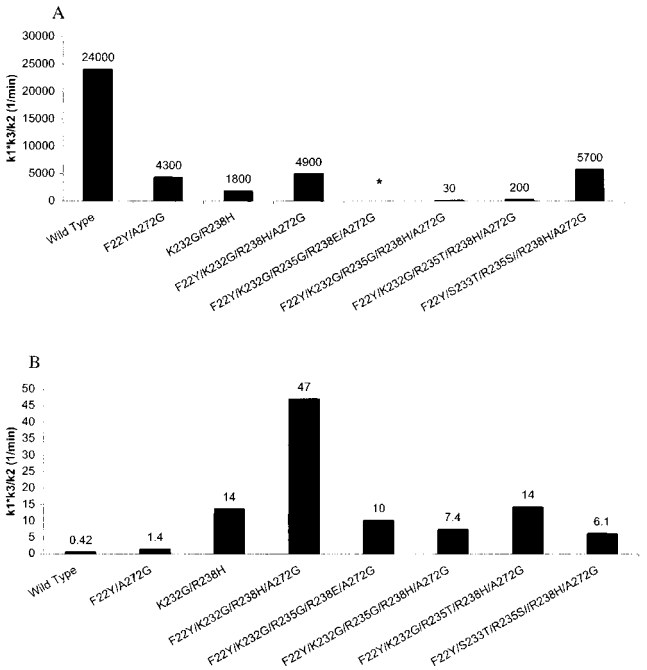

FIGURE 3: Comparison of activity with NADH and NADPH for the purified and fully characterized mutants. The value of k_1k_3/k_2 is plotted for each mutant. Panel A shows the comparison of activities with NADH, and panel B shows the comparison of NADH activities. (*) The NADPH kinetics of the F22Y/K232G/R235G/R238E/A272G mutant were not measured due to a lack of apparent activity on the native gel assay.

NADPH Kinetics. The same mutants were subjected to similar kinetic analyses using NADPH as a cofactor (Table 8). The parameters for the F22Y/K232G/R235G/R238E/A272G mutant were not determined, since there was no detectable activity with NADPH on the native gel.

The full kinetic values can be compared to the apparent kinetic parameters that have already been reported in the literature for the wild-type enzyme. Miller et al. (*13*) reported an apparent K_M for NADPH at 25 mM 2,5-DKG of 10 μM, an apparent K_M for 2,5-DKG at 0.2 mM NADPH of 26 mM, and an apparent turnover number of 500 min^{-1}. Using the full kinetic parameters, we obtain values of 22 μM, 22 mM, and 700 min^{-1}, respectively, which are very similar to previously reported values. Miller et al. also reported a 170-fold preference for NADPH over NADH at 8.5 mM 2,5-DKG and saturating cofactor. If the saturating cofactor is taken to be 0.2 mM for both NADH and NADPH, then we obtain a 190-fold preference at these concentrations. The work of Miller et al. was also done at 25 °C, but at a lower pH of 6.4.

Powers (*15*) also reported kinetic parameters for both the wild type and F22Y/A272G double mutant enzymes with NADPH as a cofactor. For the wild type, the following values were obtained: an apparent K_M of 6.4 μM for NADPH at 10 mM 2,5-DKG, an apparent K_M of 30.9 mM for 2,5-DKG at 0.2 mM NADPH, and a k_{cat} of 8.34 s^{-1}. These also compare well to our values of 13 μM, 22 mM, and 12 s^{-1}, respectively. For the F22Y/A272G double mutant, an apparent K_M of 8.2 μM for NADPH at 10 mM 2,5-DKG was reported, as compared to the value of 37 μM that was obtained from this work. Again, these values were measured at 25 °C, but Powers used a pH of 6.8. The other kinetic parameters for the F22Y/A272G double mutant were not reported by Powers due to the observation of apparent substrate inhibition above 20 mM 2,5-DKG. Substrate inhibition was not observed in this work, but substrate concentrations above 20 mM were not required to obtain the full kinetic parameters (K_b = 7.2 mM), and the 2,5-DKG substrate was purified by cation exchange (see Materials and Methods).

The results with NADPH as a cofactor were somewhat surprising. Mutants were selected for their improvements in activity with NADH, so any gains made in activity with NADPH were fortuitous. The highest k_{cat} obtained was 1100 min^{-1}, which was obtained with both the F22Y/A272G mutant and the F22Y/K232G/R235G/R238H/A272G quintuple mutant. The best value for the K_{ia} term was still seen with the wild-type enzyme at 1.1 μM, but the K232G/R238H double mutant was close to this at 8.5 μM. The dramatic increase in the K_{ia} of the F22Y/A272G mutant as compared to the wild type was unexpected. The superiority of this mutant is the result of an increased k_{cat} and a decreased K_b, which indicate a much better affinity of the enzyme−cofactor complex for the substrate. However, this benefit comes at the cost of a decreased affinity for NADPH, as indicated by the higher K_{ia}. The best value of K_a from a cofactor-binding pocket mutant was obtained with the F22Y/S233T/R235S/R238H/A272G chimeric mutant at 0.055 mM. This mutant had as well the lowest K_b value, 0.39 mM, which is 2 orders of magnitude lower than the wild-type value. However, this chimeric mutant also had the lowest k_{cat}, 130 min^{-1}.

The kinetic parameters obtained with NADPH as a cofactor can be compared to see the effects of the different mutations. The addition of the F22Y/A272G double mutant to either the wild type or the K232G/R238H double mutant produced results almost opposite to those seen with the NADH parameters (Table 6). The K_b term decreased as was seen before, but unlike with NADH, the k_{cat} and K_{ia} increased while the K_a decreased.

Table 8: Full Kinetic Parameters with NADPH as a Cofactor[a]

	k_{cat} (min^{-1})	K_{ia} (mM)	K_a (mM)	K_b (mM)
F22Y/A272G	1100 ± 80	0.035 ± 0.009	0.039 ± 0.009	7.2 ± 1.1
K232G/R238H	200 ± 20	0.0085 ± 0.0110	0.13 ± 0.02	13 ± 2
F22Y/K232G/R238H/A272G	440 ± 40	0.0098 ± 0.0025	0.058 ± 0.007	9.1 ± 0.8
F22Y/K232G/R235G/R238H/A272G	1100 ± 400	1.1 ± 0.4	0.15 ± 0.23	32 ± 19
F22Y/K232G/R235T/R238H/A272G	420 ± 30	0.23 ± 0.05	0.071 ± 0.016	8.7 ± 1.7
F22Y/S233T/R235S/R238H/A272G	130 ± 30	0.060 ± 0.026	0.055 ± 0.014	0.39 ± 0.01

[a] All reactions were performed in triplicate in 100 mM Bis-Tris, pH 7.0, at 25 °C.

Table 9: Comparison of Individual Rate Constants with NADPH as a Cofactor[a]

	k_1 (mM min^{-1})	k_2 (min^{-1})	k_3 (mM min^{-1})	k_1k_3/k_2 (min^{-1})	$(k_1k_3/k_2)/(k_1k_3/k_2)_{wt}$
wild type	16000	18	26	24000	1.0
F22Y/A272G	28000	970	150	4300	0.18
K232G/R238H	1500	13	15	1800	0.075
F22Y/K232G/R238H/A272G	7600	74	48	4900	0.20
F22Y/K232G/R235G/R238H/A272G	7300	8000	33	30	0.0013
F22Y/K232G/R235T/R238H/A272G	5900	1400	48	200	0.0083
F22Y/S233T/R235S/R238H/A272G	2400	140	330	5700	0.24

[a] $k_1 = k_{cat}/K_a$, $k_2 = k_{cat}K_{ia}/K_a$, $k_3 = k_{cat}/K_b$.

A comparison of the F22Y/K232G/R238H/A272G, F22Y/K232G/R235G/R238H/A272G, and F22Y/K232G/R235T/R238H/A272G mutants again suggests that a mutation at the 235 position does not dramatically change the geometry of the transition state, since all three have similar k_{cat}/K_b values. However, changes at this position can have large effects on the affinity of the enzyme for the cofactor, as seen in the different K_{ia} values. As was seen with NADH, the best residue at position 235 for NADPH activity appears to be the native arginine side chain (F22Y/K232G/R238H/A272G), as this led to the lowest K_{ia} and K_a.

The values of the individual rate constants, k_1, k_2, and k_3, when NADPH is used as a cofactor can also be determined (Table 9). The F22Y/S233T/R235S/R235H/A272G chimeric mutant appears to have a k_3 value of 330, which is twice as large as the F22Y/A272G double mutant. Although this mutant enzyme was not the best obtained with NADH as a cofactor, it shows a dramatic improvement in catalysis with NADPH compared to the wild-type enzyme, but the majority of the improvement occurs downstream of the rate-limiting step, which appears to be cofactor binding. The ratio of k_1k_3/k_2 shows that none of the mutants are superior to the wild-type enzyme due to its very low value of K_{ia} or k_2/k_1. However, the F22Y/A272G variant is a superior mutant at higher NADPH concentrations due to its reduced K_b and increased k_{cat}. The best mutant with NADH as a cofactor, F232Y/K232G/R238H/A272G, is also very active with NADPH. It displays a lower k_{cat} than the wild-type or F22Y/A272G enzymes, but it has a low K_{ia}, like the wild type, and a low K_b, like the F22Y/A272G double mutant. This combination results in a k_1k_3/k_2 value that is slightly greater than that seen with the F22Y/A272G double mutations (Figure 3). Therefore, improvements made when NADH is used as a cofactor do not necessarily imply significant reductions in activity with NADPH.

DISCUSSION

The engineering of nonnatural metabolic pathways shows tremendous potential for use in commercial chemical biosynthesis. A recent example is the transfer of the metabolic machinery necessary to perform polyketide synthesis into *E. coli* (*31*). This could eventually lead to the somewhat ironic notion of having industrial bacterial fermentations that produce antibiotics. Unfortunately, when the enzymes involved in the new pathway are transferred to their new environment, they may no longer be optimized to function there.

In the case of vitamin C biosynthesis, two synthetic metabolic pathways have been described. Both require an enzyme to reduce 2,5-DKG to 2-KLG. The 2,5-DKG reductase A from *Corynebacterium* is able to perform this reaction, but it requires NADPH as a cofactor. To improve this step in the metabolic pathway, we have set out to engineer the cofactor specificity to encompass the more desirable cofactor, NADH.

Mutant Production and Partial Characterization. In a previous work, we reported that single mutations at residues that interact with the 2′-phosphate group of NADPH (Figure 1) could improve activity with NADH (*1*). We have now combined the best of these mutations to try to further improve the 2,5-DKG reductase enzyme's ability to use NADH as a cofactor. We have also produced chimeric mutants that contain the same 2′-phosphate-binding loops found in other members of the AKR superfamily that have some natural affinity for NADH. The best mutants produced were then combined with the previously obtained double "up" mutant, F22Y/A272G (*15*). The cofactor specificity of the new mutants was observed through the use of activity-stained native gels.

Several of the mutants were purified and subjected to a simplified kinetic analysis with NADH as a cofactor. This information was used to select the best mutants for full kinetic characterization, and it allowed the estimation of the changes in ground- and transition-state binding energies for each new mutant. The contributions to the transition-state binding energies were compared to the contributions that were obtained with the individual component mutants. When the single mutations were combined in double and triple

mutants, the transition-state binding energies were generally not additive, as has been observed in other proteins when mutations were made at residues that interact with each other directly or indirectly (*30*). In fact, several mutants were produced that gained significant amounts of nonadditive transition-state binding energy compared to what would be expected for the contributions of the component mutations. These included the mutants K232G/R238E, K232G/R235G/R238E, and K232S/R238H, and from the native gel results, the Sl3D3βR chimeric mutants. However, when the double and triple mutants were combined with the F22Y/A272G double mutant, the free energies were additive, as expected, since residues 22 and 272 are not near the cofactor-binding pocket in the tertiary structure of the enzyme (*14*).

Full Kinetics and Comparison of Mutation Sites. The simplified kinetic analysis was extended for several of the mutants in order to obtain the full kinetic parameters for the ordered bi bi rate equation in the forward direction, in the absence of products (eq 1), using both cofactors. This information can be used to show that our results compare well to those already found in the literature, and to verify the results obtained from the simplified methods used in this work. A comparison of the full kinetic parameters with NADH to those obtained in the simplified rate equation (eq 2) showed good agreement (see Tables 3 and 6). The full kinetic parameters can also be used to obtain the mechanistic rate constants k_1, k_2, and k_3 (eq 5). Comparing the ratio of k_1k_3/k_2 to the native gel results with both cofactors also shows good qualitative agreement (see Tables 2, 4, 7, and 9).

The full kinetic parameters in the forward direction with both NADH and NADPH can be used for a detailed analysis of the effects of the mutations at the different amino acid positions. The full kinetic analysis and changes in transition-state binding energies suggest that the R238E mutation introduces a modified cofactor-binding configuration to the enzyme. The R238E mutation has previously been shown to eliminate NADPH-dependent activity and to dramatically increase the K_{ia} value when NADH is used as a cofactor (*1*). When this mutation is combined with the K232G mutation, a large nonadditive increase in transition-state binding energy is obtained. A similar increase is seen when the R235G mutation is included, as well. Although this mutated loop appears to have a reduced affinity for NADH as compared to other mutations made in this study, including the wild type, once the NADH molecule has bound, the enzyme−cofactor complex exhibits the highest specificity constant (k_{cat}/K_b) of any mutant studied for the substrate. This suggests that the cofactor binds with a slightly different geometry, which improves the configuration of the substrate-binding pocket. The specificity constant (k_{cat}/K_b) was further increased by the addition of the F22Y/A272G double mutations.

Mutations at the Arg235 position exhibit the opposite effect. Upon comparing the quadruple mutant F22Y/K232G/R238H/A272G with the quintuple mutants containing R235G and R235T, it appeared that mutations at the Arg235 position had little effect on the transition state of the enzyme, as evidenced by similar k_{cat}/K_b values. However, mutations at this position did have a large effect on the affinity of the enzyme for either cofactor. The role of Arg235 in directly mediating the affinity of the enzyme for its cofactor has not previously been reported in the 2,5-DKG reductase literature.

The R238H amino acid substitution was previously shown to result in the largest single improvement in activity with NADH. We have proposed that this side chain may produce a favorable stacking interaction with the adenine ring of the bound NADH molecule (*1*). In this work, this mutation was used to form superior double and triple mutants. Interestingly, these mutants also retained a high degree of activity with NADPH as a cofactor. Work with the R3αHSD from rat liver has shown that the arginine at 276 (analogous to Arg238) acts as an anchor for the 2′-phosphate of NADPH (*11*). When protonated, the R238H mutation is fairly conservative. Presumably, the histidine side chain can also promote the binding of NADPH with a stacking interaction, but further studies including an analysis of pre-steady-state kinetics would be required to see if the histidine side chain is also able to anchor the 2′-phosphate of NADPH.

The F22Y/S233T/R235S/R238H/A272G quintuple mutant was made by combining the 2′-phosphate-binding pocket from the 3-dehydroecdysone 3β-reductase from *Spodoptera littoralis* (S233T/R235S), the best single mutant made in the previous mutagenesis work (R238H), and a pair of mutants that were previously used to improve NADPH activity (F22Y/A272G). Combined, they produced improvements in activity with NADH, but their effect on activity with NADPH was more interesting. Although these mutations resulted in a decreased k_{cat} value, a striking decrease in the K_b value for NADPH to 2 orders of magnitude below that of the wild-type enzyme was observed. This improvement is largely responsible for the enzyme having a k_1k_3/k_2 value that is below the wild type, but higher than the F22Y/A272G double mutant by itself. This quintuple mutant may be of value in an intracellular environment where both cofactors are present, and the substrate concentration is very low.

Most Active Mutant Comparisons. The K232G/R238H mutant had the lowest K_{ia} value of any mutant studied. When these mutations were combined with the F22Y/A272G double mutant, improvements in other kinetic parameters resulted in the F22Y/K232G/R238H/A272G mutant being the most active with NADH as a cofactor (Figure 3). This mutant showed the highest increase in transition-state binding energy, and, fortuitously, the gains made in activity with NADH for this mutant did not lead to a serious loss in activity with NADPH. A comparison of the ratio of the rate constants k_1k_3/k_2 showed that the mutant was 110-fold improved over the wild type with NADH and had similar activity with NADPH as did the F22Y/A272G double mutant.

This quadruple mutant was the highest activity mutant produced in this work, and the activity of this enzyme with NADH can be compared to that of the wild type with NADPH. If only the values of k_1k_3/k_2 are compared, the wild-type enzyme is 5700 times more active with NADPH than with NADH. However, the wild type is only 51 times more active with NADPH than is the quadruple mutant with NADH.

Knowledge of the full kinetic parameters with both cofactors can be used to compare the performance of the mutant and wild-type enzymes at specific conditions (Figure 4). Another way to compare the enzymes is to examine them under saturating conditions. If cofactor concentrations are

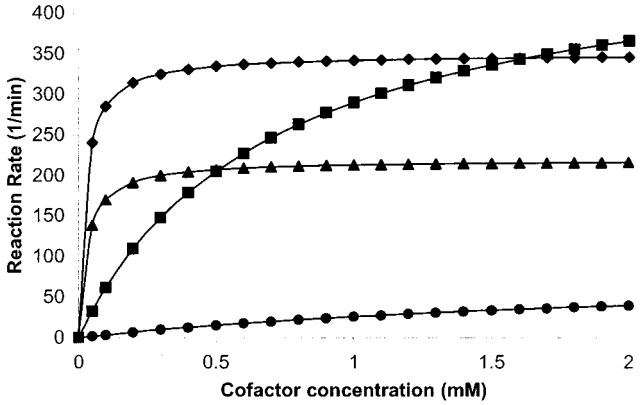

FIGURE 4: Predicted initial reaction rates (dP/dt, E_t^{-1}) (eq 1) of the wild-type and F22Y/K232G/R238H/A272G mutant enzymes with NADH or NADPH at 100 mM 2,5-DKG. The wild type with NADPH is shown as (◆), wild type with NADH is shown as (●), the quadruple mutant with NADPH is shown as (▲), and the quadruple mutant with NADH is shown as (■).

taken to be very large, then the ordered bi bi equation reduces to

$$\frac{dP}{dt} = \frac{E_t k_{cat} B}{K_b + B} \quad (6)$$

Comparing the values of k_{cat}/K_b for the wild type with NADPH (26 min^{-1} mM^{-1}) and the quadruple mutant with NADH (31 min^{-1} mM^{-1}) shows that, at high cofactor concentrations, the quadruple mutant with NADH would be *more active* than the wild type with NADPH (Figure 4). If both cofactor and substrate are saturating, then k_{cat} for the quadruple mutant with NADH is 990 min^{-1} as compared to 700 min^{-1} for the wild type with NADPH.

This engineered quadruple mutant catalyst may eventually be able to improve both in vitro and in vivo vitamin C biosynthesis schemes. In the intracellular environment, the enzyme would no longer be constrained to use just the available NADPH; the ability to use both cofactors might be beneficial both for cellular growth and for 2-KLG production. In the in vitro bioreactor, the production of 2-KLG could be catalyzed with either cofactor, or with both simultaneously, and concentrations of enzymes and cofactors could be optimized for maximal productivity and cost-effectiveness.

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