Use of Structural Comparisons To Select Mutagenic Targets in Aspartate-β-semialdehyde Dehydrogenase†

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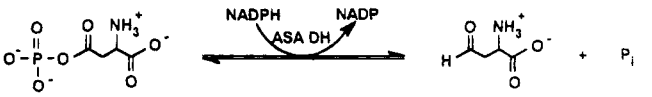
ABSTRACT: L-Aspartate-β-semialdehyde dehydrogenase (ASA DH) from *Escherichia coli* has been probed by site-directed mutagenesis to identify residues that play an important function in the catalytic activity of the enzyme. Sequence homology searching among ASA DHs that have been isolated from other species and comparisons with the structures of functionally similar D-glyceraldehyde-3-phosphate dehydrogenases (GAPDH) that have been solved from several species have been utilized to select appropriate targets for mutagenesis. A highly conserved active site glutamine has been identified in the *E. coli* ASA DH that enhances the reactivity of the enzyme. Alteration of this residue leads to an enzyme with reduced catalytic efficiency, yet with an unchanged binding affinity for substrates and coenzyme. Replacement of an arginine residue that is conserved throughout the ASA DH and GAPDH enzyme families leads to a significant decrease in catalytic turnover and is the only mutation examined that also results in a decreased affinity for the substrates of the reaction. This residue is assigned a role in the binding of the substrate aspartate-β-semialdehyde. Sequence alignment of ASA DH with other NADP- and NAD-dependent enzymes has resulted in the identification of a putative pyridine nucleotide binding region. Substitution of two amino acids in this region with neutral or positively charged side chains has resulted in a change in enzyme specificity. For wild-type ASA DH, NADP is strongly favored as the coenzyme, while in this mutated enzyme the selectivity has been lowered by a factor of 60, and this enzyme has comparable affinities for either pyridine nucleotide.

L-Aspartate-β-semialdehyde dehydrogenase (EC 1.2.1.11) (ASA DH)[1] is one of the three enzymes in the common pathway starting from L-aspartic acid and leading out to the formation of L-lysine, L-isoleucine, L-methionine, and L-threonine in *Escherichia coli*. The first and third steps in this pathway are catalyzed by the bifunctional enzymes aspartokinase-homoserine dehydrogenase I and II. Another monofunctional enzyme, aspartokinase III, also catalyzes the initial reaction in the pathway. ASA DH catalyzes the intervening branch point reaction between the aspartokinase and the homoserine dehydrogenase reactions, with one of the branches leading to the production of L-lysine and the other leading to L-methionine, L-isoleucine, and L-threonine (Cohen, 1983). In the biosynthetic direction, ASA DH catalyzes the formation of L-aspartate-β-semialdehyde (L-ASA) by the reductive dephosphorylation of L-β-aspartyl phosphate utilizing NADPH (Scheme 1).

ASA DH is encoded by the *asd* gene in *E. coli*. The nucleotide sequence of the gene has been determined, and the primary structure of the corresponding enzyme has been deduced (Haziza et al., 1982). ASA DH exists as a homodimer, with each subunit containing 367 amino acids and a molecular weight of 39 950 calculated from the amino acid sequence. The *asd* gene has been cloned into an expression system to facilitate enzyme purification and characterization (Preiss et al., 1982; Karsten & Viola, 1992). The kinetic mechanism of ASA DH has been studied by

Scheme 1: Reaction Catalyzed by Aspartate-β-semialdehyde Dehydrogenase



Karsten and Viola (1991), and a partial random sequential mechanism with a preferred order for coenzyme binding has been determined. Chemical modification and site-directed mutagenesis studies have also been employed to examine the chemical mechanism of the enzyme (Karsten & Viola, 1991, 1992). An essential cysteine, cysteine 135, has been identified from these studies. This sulfhydryl group acts as a nucleophile, attacking the β-carbonyl group of the phosphorylated amino acid substrate to initiate catalysis. Crystallization of *E. coli* ASA DH has been carried out, and the preliminary examination of these crystals has been reported Kryger et al., 1992). However, the complete three-dimensional structure of the enzyme has not yet been solved.

Our interests have focused on an examination of the active site amino acids that are involved in catalysis and/or substrate and coenzyme binding. Additional functional residues, besides the nucleophilic sulfhydryl group, must be identified in order to ascertain the detailed chemical mechanism of this enzyme. Several additional amino acid targets have been selected, based on a comparative study of homologous sequences between ASA DH and the functionally and mechanistically related glyceraldehyde-3-phosphate dehydrogenases (GAPDH) from various species (Biellmann et

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[1] Abbreviations: L-ASA, L-aspartate-β-semialdehyde; ASA DH, L-aspartate-β-semialdehyde dehydrogenase; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase.

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al., 1980; Holland & Westhead, 1973). Comparison of the amino acid sequences from both enzyme families has revealed a reasonably good alignment in several regions. Several mutations of ASA DH have been carried out by site-directed mutagenesis, and the properties of these mutated enzymes have been compared to those of the wild-type enzyme. These results suggest that the targeted amino acids are functionally important for the catalytic activity of ASA DH and support the judicious use of sequence homology comparisons for the identification of critical amino acid residues.

MATERIALS AND METHODS

Materials. Oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA), and the oligonucleotide-directed mutagenesis kit was obtained from Bio-Rad Laboratories (Hercules, CA). The DNA sequencing kit was purchased from U.S. Biochemical (Cleveland, OH), and [α-^{35}S]dATP was obtained from DuPont. L-Allylglycine was purchased from Sigma Chemical Co. (St. Louis, MO), and L-aspartic-β-semialdehyde (L-ASA) was prepared by the ozonolysis of L-allylglycine according to the method of Black and Wright (1955). All other reagents were from common commercial sources and were used without further purification.

Protein Sequence and Alignment. The amino acid sequences of the proteins shown in this paper were retrieved from either GenBank or SWISS-PROT databases *via* the electronic mail server at the National Center for Biological Information of the National Library of Medicine. Multiple sequence alignments were performed using the program Clustal V (Higgins et al., 1992).

Mutagenesis and Isolation of Mutated Enzymes. Site-directed mutagenesis of the *asd* gene was carried out as described earlier (Karsten & Viola, 1992). Oligonucleotides were designed to generate specific site-directed mutations while at the same time to either create or eliminate a cleavage site for a restriction endonuclease. All of the mutations have been verified by restriction enzyme digestion and by DNA sequencing of the regions that contain the mutations using the dideoxynucleotide method (Sanger et al., 1977). Production and purification of the wild-type and mutant enzymes were performed by the method of Karsten and Viola (1991), except that YT media was used in place of minimal media and the heat treatment purification step was omitted. Each of the mutated enzymes were readily purified by the same method that is used for the wild-type enzyme, indicating that no gross conformational changes of the enzyme has occurred upon mutation. The purity of the proteins was judged by SDS−PAGE gels (Laemmli, 1970) stained with Coomassie blue. Protein concentrations were determined by the method of Bradford (1976).

Enzyme Assays and Data Analysis. Enzyme assays were run on a Perkin-Elmer Lambda 1 spectrophotometer that is equipped with a thermostated cell holder connected to a circulating water bath. Initial velocities were measured based on the appearance of NADPH absorbance at 340 nm. Assays were conducted at 25 °C in a total volume of 1 mL containing 200 mM CHES buffer (pH 8.6), 1 mM EDTA, 1 mM DTT, and from 0.055 to 3.8 μg of ASA DH. In most cases, the kinetic parameters were determined at saturating concentration of the other substrates. For mutated enzymes, where saturation with L-ASA was impractical due to the high acidity of the stock solution, the concentrations of both the substrate and the coenzyme were varied. These data were then fitted to the equation for a sequential mechanism. BASIC versions (Viola, 1989) of Cleland's kinetic programs (1967) were utilized to determine the kinetic parameters.

Enzyme inactivation studies were conducted at the indicated pH values in a reaction mixture containing 200 mM buffer, 1 mM NADP, 50 mM phosphate, and 50 μM NEM. Enzyme concentrations were 0.21 μM for the wild-type and 2.7 μM for the mutant enzymes. The pH was maintained with MES from 5.8 to 6.6, HEPES from 7.1 to 8.4, CHES from 8.6 to 9.8, and CAPS from 10.2 to 10.6. Aliquots were removed at defined time intervals, and the remaining activity was assayed as described above. The data were fitted by a least squares fitting algorithm to a model for a titration curve (eq 1) that assumes a change in the rate of inactivation of a single group as a function of pH:

$$k_{inact} = \frac{k_{HA} + k_A K_a/[H^+]}{1 + K_a/[H^+]} \quad (1)$$

where k_{inact} is the observed rate of inactivation, k_{HA} and k_A are the limiting rates of inactivation of the protonated and ionized forms of the functional group, respectively, and K_a is the ionization constant of the enzymatic group that is modified.

Binding Studies. The binding of pyridine nucleotides to ASA DH was measured by fluorescence quenching on an SPF-500 spectrofluorimeter. Enzyme solutions (subunit concentrations of 2.2−3.8 μM) were prepared in 50 mM HEPES buffer, pH 7, and nucleotide concentrations were varied from 2 to 60 μM. The enzyme solutions were excited at 285 nm, and the fluorescence emission intensities were measured at 328 nm, with the slit widths on the excitation and emission monochromators set at 2 and 4 nm, respectively.

RESULTS

Selection of Mutation Sites. The choice of the appropriate amino acid targets for mutagenesis was based on sequence comparisons among related enzymes and the hypothesis that proteins of similar function have similar tertiary structure (Rossmann et al., 1975). The amino acid sequences of ASA DH from various sources were aligned together with several GAPDH sequences using Clustal V program. GAPDH was chosen as a model enzyme due to its functional similarity to ASA DH and because the high-resolution three-dimensional structure of the enzyme from several sources, including *Bacillus stearothermophilus* (Skarzynski et al., 1987), lobster (Murthy et al., 1980) and human muscle (Mercer et al., 1976), has been solved. The ASA DH sequence was also compared with other NADP-utilizing and also NAD-utilizing enzymes to aid in the identification of the coenzyme binding site (Wierenga et al., 1985, 1986).

Mutation of a Catalytic Residue. The alignment between the ASA DH and GAPDH enzyme families indicates the presence of a highly conserved core region in both groups (Figure 1) from valine 131 to alanine 163 (the position numbers are those of the *E. coli* ASA DH), including the strictly conserved cysteine that has been shown to be an

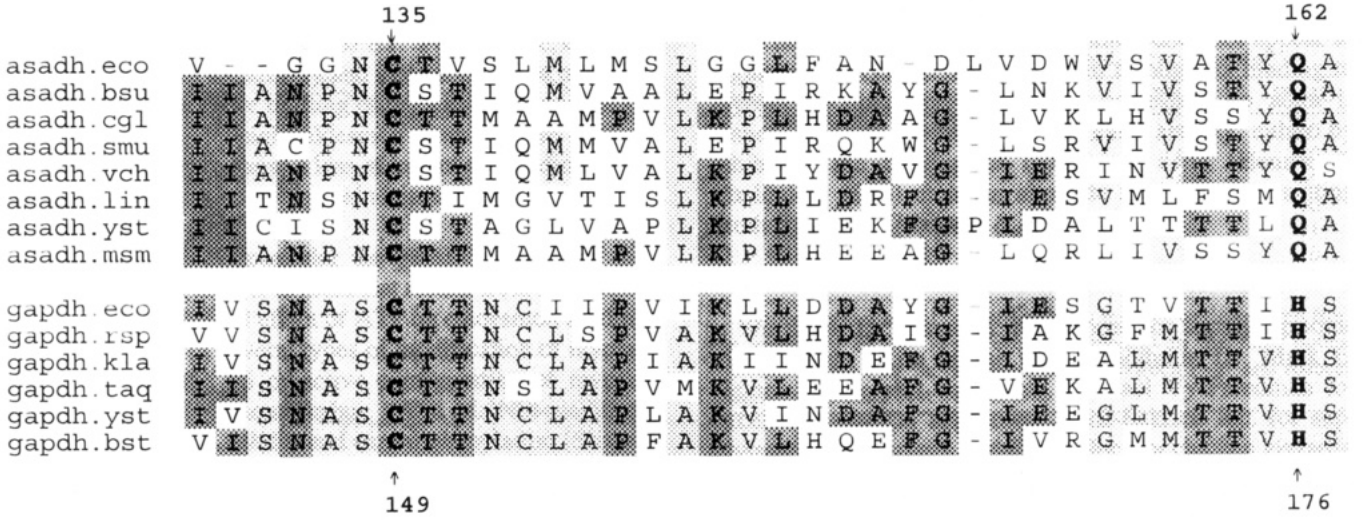

FIGURE 1: Alignment of ASA DH and GAPDH core regions. The position numbers on top of each block are those of *E. coli* ASA DH, while the numbers at the bottom are those of *B. stearothermophilus* GAPDH. The strictly conserved residues across both enzyme families are enclosed in the dark shaded box, while the residues that are only conserved within each family are enclosed in the light shaded box. The catalytic cysteine (positions 135 or 149) and the conserved glutamine (162) and histidine (176) are highlighted. The species abbreviations are as follows: eco, *Escherichia coli*; bsu, *Bacillus subtilis*; cgl, *Corynebacterium glutamicum*; smu, *Streptococcus mutans*; vch, *Vibrio cholerae*; lin, *Leptospira interrogans*; yst, yeast (*Saccharomyces cerevisiae*); msm, *Mycobacterium smegmatis*; rsp, *Rhodobacter sphaeroides*; kla, *Kluyveromyces lactis*; taq, *Thermus aquaticus*; bst, *Bacillus stearothermophilus*.

Table 1: Kinetic Parameters of Mutated ASA DH[a]

		1-ASA		phosphate	
enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
WT	610 ± 20	0.17 ± 0.02	3600 ± 400	4.8 ± 0.8	130 ± 20
Q162N	47 ± 2	0.15 ± 0.01	310 ± 30	4.6 ± 1.1	10 ± 2
Q162H	177 ± 10	0.25 ± 0.03	710 ± 90	4.7 ± 0.5	38 ± 5
Q39D	290 ± 20	0.13 ± 0.01	2300 ± 200	4.7 ± 0.6	62 ± 9
R267L	58 ± 4	5.3 ± 0.4	11 ± 1	19 ± 2	3.0 ± 0.3

[a] Reaction conditions: 200 mM CHES buffer (pH 8.6), 1 mM DTT, 1 mM EDTA, and 0.055–3.8 μg/mL enzyme, 25 °C.

essential amino acid for catalysis in both enzymes. Of the 32 residues in this region, 12 are conserved in most of the members of these families. A conserved glutamine (Gln 162) in the ASA DH family is well aligned with a histidine (His 176 of *B. stearothermophilus* GAPDH) that is also conserved in the GAPDH family. This histidine has been shown to be an active site catalyst that enhances the reactivity of the thiol group of the cysteine nucleophile of GAPDH through ion pairing (Harrigan & Trentham, 1973; Polgar, 1975). Accordingly, glutamine 162 of *E. coli* ASA DH has been altered to asparagine 162 to investigate its role in ASA DH catalysis. The Q162N mutation shows no significant changes in the K_m values for the substrates and the coenzymes. However, this mutation leads to a 13-fold decrease in k_{cat} for the reaction examined in the oxidative phosphorylation direction (Table 1). A histidine was also substituted at this position to determine if the catalytic residue of GAPDH could be productively inserted into the ASA DH active site. The k_{cat} for this mutant does increase by nearly 4-fold when compared to the asparagine 162 mutant. However, this value is still less than 30% that of the wild-type enzyme (Table 1).

ASA DH has previously been shown to be inactivated by sulfhydryl reagents such as *N*-ethylmaleimide (NEM) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Karsten & Viola, 1991). The Q162N enzyme is also inactivated by NEM but shows increased sensitivity to the reagent. When examined at pH 7.0, the rate of inactivation of the wild-type enzyme

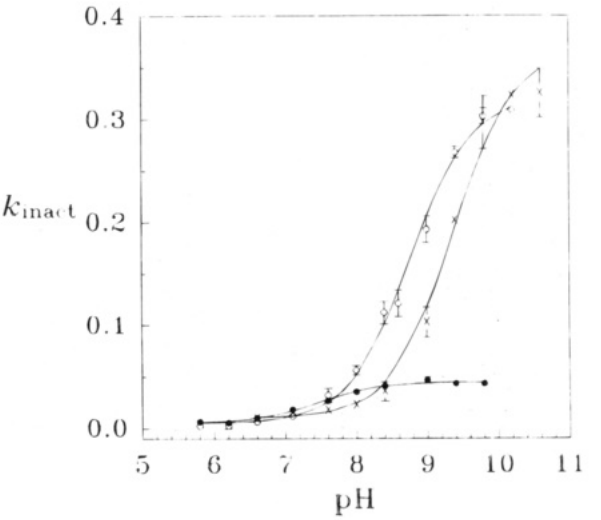

FIGURE 2: pH dependence of the inactivation of ASA DH by *N*-ethylmaleimide. Inactivation studies were conducted at 25 °C at the pH values indicated in a reaction mixture containing 200 mM buffer, 1 mM NADP, 50 mM P_i, and 50 μM NEM. The rate of inactivation was measured at each pH for the wild-type enzyme (●), the Q162H mutant (○), and the Q162N mutant (×). The curves are a least squares fit to the data to a model that assumes a change in the rate of inactivation as a result of the ionization of a single reactive group.

by NEM in the absence of coenzyme is 0.6 min^{-1} mM^{-1}, while the Q162N enzyme is inactivated at 1.9 min^{-1} mM^{-1}. A comparison of the pH dependence of NEM inactivation shows that the Q162N mutant becomes considerably more sensitive to NEM at higher pH (Figure 2). The p*K* of the cysteine that reacts with NEM is shifted from 7.6 for the wild-type enzyme (Karsten & Viola, 1991) to 9.3 for the mutated enzyme. A similar increase in sensitivity to NEM is observed for the Q162H mutant, with the cysteine p*K* value shifted to 8.6 (Figure 2). The pH profile for the enzyme-catalyzed reaction is however unchanged from that of the wild-type enzyme (data not shown).

Mutation of a Substrate Binding Residue. An arginine residue (Arg 267) has also been identified that is strictly

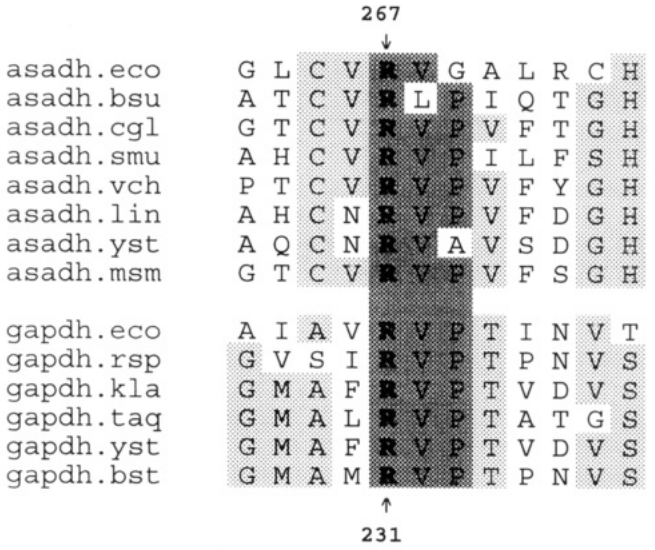

FIGURE 3: Homology between the ASA DH and the GAPDH family of enzymes in the region around an active site arginine. The designation of the shaded regions and the species abbreviations are the same as in Figure 1. The active site arginine is highlighted.

conserved among both enzyme families (Figure 3). For the *B. stearothermophilus* GAPDH, the side chain of this arginine has been shown to interact with the substrate, glyceraldehyde-3-phosphate, *via* hydrogen bonding to the phosphate (Skarzynski et al., 1987). Substitution of arginine 267 by leucine was carried out to determine if this functional group plays a similar role in ASA DH catalysis. Conversion of this invariant residue generates a mutated enzyme with drastically altered kinetic parameters. The K_m value for L-ASA increases by 30-fold, while the k_{cat}/K_{ASA} value drops by 330-fold as compared to the wild-type enzyme (Table 1). NADP and phosphate also display less favorable k_{cat}/K_m values, 120- and 40-fold smaller than that of the wild-type enzyme, respectively.

Mutation in the Coenzyme Binding Region. Additional mutagenic targets have been selected based on the proposed fingerprint sequence for a pyridine nucleotide coenzyme binding domain (Wierenga et al., 1985, 1986). The conserved glycine residues at positions 8, 11, and 14 suggest that the first 40 amino acids at the amino terminus can form a portion of the NADP binding site of ASA DH. This suggestion is supported by the observation that this region is reasonably well aligned with the NAD and NADP binding domains that have been identified in other pyridine nucleotide-utilizing enzymes (Figure 4). Wild-type ASA DH can utilize NAD instead of NADP as the coenzyme. However, the k_{cat} is 140-fold lower and K_{NAD} is 65-fold higher than K_{NADP}. Conserved arginines located within the NADP binding domain have been proposed to be involved in binding to the 2′-phosphate of the pyridine nucleotide (Wierenga et al., 1985). This role was examined in ASA DH by altering the conserved arginine 31 to a leucine. The kinetic parameters for this R31L mutant are unchanged with NADP as the coenzyme. However, the selectivity (k_{cat}/K_m) for NAD does show a modest 4-fold increase for this mutated enzyme.

A nonconserved glutamine (Gln 39) in the *E. coli* enzyme is replaced by an arginine in the ASA DH enzymes that have been sequenced from other sources. This glutamine was mutated to aspartic acid in an attempt to alter the coenzyme specificity of ASA DH from an NADP-requiring to an NAD-requiring enzyme. The mutant Q39D retains almost 50% activity when NADP is utilized as the cofactor; however, there is a 10-fold increase in K_{NADP} observed compared with the wild-type enzyme (Table 2). The affinity of the Q39D enzyme for NAD does not increase; K_{NAD} is actually higher by a factor of 2. However, the k_{cat} for NAD compared to NADP increases for a factor of 10.

To determine whether both of these positions, 31 and 39, are involved in a coordinated fashion in interactions with NADP, a double mutant was prepared in which the naturally

NADP-dependent enzymes

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                          8  11 14                    31        39
                          ↓  ↓  ↓                     ↓         ↓
asadh.eco        VGFIGWRGMVGSVLMQRMVEERDF--DAIRPVFFSTSQL
asadh.smu        VAIVGATGAVGTRMIQQL-EQSTLPVDKVR--LLSSSRS
asadh.cgl        IAVVGATGQVGQVMRTLL-EERNFPADTVR--FFASPRS
gthrd.eco        RVAVVGAGYIAVELAGVINGLGAKTHLFVR--KHAPLRS
mecrd.sau        RLAVIGSGYIAAELGQMFHNLGTEVTLMQR--SERLFKT
homdh.cgl        GIALLGFGTVGTEVMRLMTEYGDE--LAHRIGGPLEVRG
maldh.ptr        TFLFLGAGEAGTGIAELIALEMSRR---SKTPLEETRKK
carrd.hum        TGGNKGIGLAIVRDLCRLFS--GDVVLTAR--DVTRGQA
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NAD-dependent enzymes

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gapdh.taq        KVGINGFGRIGRQVFRILHSRGVEVALINDLTDNKTLAH
alcdh.hum        TCAVFGLGGVGLSAVMGCKAAGAARIIAVDINKDKFAKA
dhldh.yst        RLTIIGGGIIGLEMGSVYSRLGS-KVTVVEFQPQIGASM
fmadh.cam        NVAVFGGGIVGLSVIQGCAERGAAQIILVDISDKKEEWG
glydh.csa        TVGVIGAGRIGSAYARMMVEGFKMNLIYFDLYQSTRLEK
hicdh.lca        TVGVMGTGHIGQVAIKLFKGFGAKVIA-YDPYPMKGDHP
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FIGURE 4: Alignment of the NADP and NAD binding domains of some pyridine nucleotide utilizing enzymes. Enzyme abbreviations are as follows: asadh, aspartate-β-semialdehyde dehydrogenase; alcdh, alcohol dehydrogenase; carrd, carbonyl reductase; dhldh, dihydrolipoamide dehydrogenase; fmadh, formaldehyde dehydrogenase; gapdh, glyceraldehyde-3-phosphate dehydrogenase; glydh, glycerate dehydrogenase; gthrd, glutathione reductase; hicdh, D-2-hydroxyisocaproate dehydrogenase; homdh, homoserine dehydrogenase; maldh, malate dehydrogenase; mecrd, mercuric reductase. Species abbreviations are as follows: cam, *Candida maltosa*; cgl, *Corynebacterium glutamicum*; csa, *Cucumis sativus*; eco, *Escherhicia coli*; hum, human; lca, *Lactobacillus casei*; ptr, *Populus trichocarpa*; sau, *Staphylococcus aureus*; smu, *Streptococcus mutans*; taq, *Thermus acquaticus*; yst, yeast.

Table 2: Pyridine Nucleotide Coenzyme Specificity of Mutated ASA DH[a]

	NADP			NAD			NADP/NAD ratio		
enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat}	$1/K_m$	k_{cat}/K_m
WT	680 ± 50	0.19 ± 0.02	3600 ± 500	4.8 ± 0.4	12 ± 1	0.39 ± 0.05	141 ± 15	65 ± 9	9200 ± 1700
Q162N	55 ± 3	0.21 ± 0.03	260 ± 40	0.53 ± 0.05	14 ± 2	0.038 ± 0.006	103 ± 11	66 ± 12	6800 ± 1500
R31L	890 ± 10	0.23 ± 0.01	3900 ± 200	14 ± 1	9.5 ± 1.0	1.5 ± 0.2	64 ± 6	41 ± 5	2600 ± 400
Q39D	270 ± 9	1.7 ± 0.1	160 ± 10	19 ± 3	25 ± 4	0.76 ± 0.17	14 ± 2	15 ± 3	210 ± 50
R31D/Q39D	270 ± 20	1.4 ± 0.2	190 ± 30	3.6 ± 0.1	2.8 ± 0.3	1.3 ± 0.2	74 ± 4	2.0 ± 0.3	150 ± 30

[a] Reaction conditions: 200 mM CHES buffer (pH 8.6), 1 mM DTT, 1 mM EDTA, and 0.055−3.8 μg/mL enzyme, 25 °C.

occurring amino acids in these positions were replaced with aspartic acids. The kinetic parameters of this double mutant for L-ASA and phosphate are unchanged from those of the Q39D single mutant. The parameters with NADP as the coenzyme are also unchanged from the single mutant (Table 2). With NAD as the coenzyme, k_{cat} decreases by 5-fold; however, K_{NAD} becomes 10-fold tighter, leading to a net 2-fold improvement in k_{cat}/K_m for NAD. The binding of NAD and NADP to the wild-type enzyme and the double mutant were determined by fluorescence quenching studies. The K_d of NADP for the wild-type enzyme is 7.3 ± 0.2 μM, while the binding of NAD to the wild-type enzyme was too weak to reliably measure by this method. For the double mutant, the affinity for NADP decreases by a factor of 3 to 25.1 ± 1.1 μM, while the affinity for NAD increases to 20.4 ± 1.8 μM. Further, the selectivity (k_{cat}/K_m) for NADP compared to NAD decreases from over 9000 in the wild-type enzyme to about 150 in the double mutant.

DISCUSSION

It has been shown that both GAPDH and ASA DH catalyze the oxidative phosphorylation of an aldehyde substrate, and both possess an essential cysteine residue that is involved in the enzymatic reaction. It has also been postulated that both enzymes carry out this reaction by a chemical mechanism consisting of hemithioacetal formation, followed by hydride transfer, and subsequent phosphorolysis of the acyl enzyme (Harris & Waters, 1976; Karsten & Viola, 1991). Moreover, it has been previously reported that both enzymes form relatively stable acyl enzyme intermediates in the absence of an acyl acceptor (Biellmann et al., 1980; Seydoux et al., 1973). The similarity in properties between these enzymes has been used to extrapolate information available from sequence homology and the three-dimensional structure of GAPDH to aid in the selection of mutagenic targets in ASA DH.

Identification of an Active Site Catalyst. Histidine 176 has been proposed to be an active site catalyst in GAPDH (Buehner et al., 1973). In the high-resolution structure of *B. stearothermophilus* GAPDH, Skarzynski et al. (1987) describe histidine 176 as forming a hydrogen bond to the carbonyl oxygen atom of the adjacent serine 177 at a distance of 2.63 Å. In this structure, determined in the absence of the substrate, histidine 176 is situated about 3.6 Å from sulfur atom of cysteine 149, the active site nucleophile. Nevertheless, the significance of this histidine residue in enhancing the reactivity of the cysteine thiol group has been verified by site-directed mutagenesis (Soukri et al., 1989), which showed a decrease in k_{cat} to 2% that of the wild-type enzyme when histidine 176 was converted to an asparagine. This H176N mutant has a decreased sensitivity to the sulfhydryl reagent iodoacetamide at neutral pH and a shift in the pK value of the active site cysteine from 8.6 to 9.3 when histidine is replaced.

Sequence alignment between the GAPDH and the ASA DH enzyme families has indicated that the corresponding position in ASA DH is occupied by an invariant glutamine (Figure 1). A subtle alteration has been made at this position (glutamine → asparagine), with no discernable effect on the Michaelis constants for any of the substrates. This confirms that the native structure of ASA DH has not been perturbed by this substitution. However, the catalytic efficiency of the Q162N enzyme has decreased to only 8% that of the wild-type enzyme. This observation implicates glutamine 162 in catalysis rather than in substrate or coenzyme binding. Since the side chain functional group of glutamine is uncharged, the available mode of interaction with adjacent amino acids is most likely to involve hydrogen bonding. In the absence of backbone structural rearrangements, substitution of an asparagine at this position could alter the location of the amide functional group by about 2.5 Å. This displacement would certainly be sufficient to disrupt any existing hydrogen bonding interactions either to cysteine 135 or to the acyl group that is covalently attached to the enzyme as an intermediate in the reaction.

ASA DH is inactivated by the sulfhydryl reagent NEM, although the rate of inactivation is fairly slow, even at higher pH where cysteine 135 is fully ionized (Karsten & Viola, 1991). Both Q162N and Q162H enzymes show enhanced sensitivity to NEM in the absence of coenzyme even at neutral pH, and they are inactivated at a rate that is 7-fold faster than that of the wild-type enzyme at higher pH. This may be a consequence of the enhanced accessibility of the reagent to the active site cysteine when glutamine is replaced by either asparagine or histidine. The pK value of the native cysteine nucleophile increases from 7.6 to 8.6−9.3 in these mutant enzymes. This increase is the same trend that was observed in GADPH when the catalytic histidine was replaced (Soukri et al., 1989) and supports the assignment of glutamine 162 as an active site catalyst in the ASA DH reaction.

Identification of a Residue Involved in Substrate Binding. In the holoenzyme structure for *B. stearothermophilus* GAPDH (Skarzynski et al., 1987), arginine 231 has been assigned the role of binding to the C_3-phosphate of glyceraldehyde 3-phosphate. This contribution is apparent only when the coenzyme NAD is bound to the enzyme. Arginine 267 in ASA DH has been found to align wth arginine 231 in GAPDH (Figure 3), suggesting that this residue may play an equivalent role in substrate binding in ASA DH. Replacement of arginine 267 with leucine eliminates the positive charge at this position, which would disrupt the potential

ion pairing between the α-carboxyl group of L-ASA and the guanidinium group at the binding site. Among the mutations that have been examined, the R267L enzyme is the only case where the Michaelis constants for the substrates have been dramatically altered. K_{ASA} has increased over 30-fold (Table 1), with smaller but still substantial increases in K_{P_i} and K_{NADP}.

Alteration of Coenzyme Specificity. Alignment of the sequence of ASA DH with those of other pyridine nucleotide-requiring enzymes has led to the identification of an amino terminal region that appears to be involved in coenzyme binding (Figure 4). The primary structure of ASA DH at this NADP binding region does not obey the nearly universal motif pattern (Wierenga et al., 1985), *i.e.*, the sequence of Gly-X-Gly-X-X-Gly is not observed in this enzyme. Instead, a pattern of Gly-X-X-Gly-X-X-Gly is observed from Gly 8 to Gly 14 (Figure 4). This provides another example of deviation from the nucleotide-binding fingerprint sequence (Rossmann et al., 1974). For NAD-dependent enzymes, an invariant acidic residue (Asp 32 in *B. stearothermophilus* GAPDH) has been shown to be involved in hydrogen bonding with the 2′-hydroxyl group of the adenine ribose (Wierenga et al., 1985). For NADP-specific enzymes, the residue at this position is replaced by a small, neutral side chain (Branden & Tooze, 1991) or, in some cases, by a positively charged functional group that interacts with the 2′-phosphate group (Wierenga et al., 1985).

ASA DH utilizes NADP as its coenzyme *in vivo*, although the enzyme is capable of turning over NAD to NADH *in vitro* at a very low catalytic efficiency. The replacement of arginine 31 and glutamine 39 with aspartic acids leads to a 10-fold decrease in K_m and a 3-fold decrease in affinity for NADP, which indicates that there is now an electrostatic repulsion between the charged carboxylate groups of these aspartates and the 2′-phosphate group of NADP. This double mutant now has a slightly higher binding affinity for NAD over NADP; however, this mutant still shows a favorable k_{cat}/K_m for NADP over NAD by 150-fold. This implies that there are additional residues in this domain that interact with the 2′-phosphate and thereby contribute to NADP specificity. Alteration of only one or two amino acids has not been sufficient to reverse the coenzyme specificity in several enzyme systems that have been examined (Clermont et al., 1993; Perham et al., 1991; Fan et al., 1991). The removal of four positively charged amino acids is required in order to reverse the specificity of keto acid reductoisomerase from an NADPH-specific to an NADH-specific enzyme (Rane and Calvo, personal communication). In the case of glutathione reductase, the replacement of two arginines in the coenzyme binding domain dramatically lowers the selectivity of the enzyme for NADPH, although this double mutant still shows a preference for its physiological coenzyme over NADH (Scrutton et al., 1990). Only when five additional amino acids were mutated, using the NADH binding domain of dihydrolipoamide dehydrogenase as a template, was the coenzyme specificity of glutathione reductase reversed (Scrutton et al., 1990). Nevertheless, the results that have been reported here clearly support the hypothesis that arginine 31 and glutamine 39 participate in determining the specificity of *E. coli* ASA DH for NADP.

The identification of amino acid residues that are conserved within a family of functionally related enzymes is not sufficient to permit their assignment to a catalytic role. However, if some additional information is available, such as a high-resolution structure of a related enzyme or the presence of a well-characterized structural motif, then structural comparisons can be instrumental in the selection of amino acid targets for further investigation.

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