

# Expression and Purification of Aspartate $\beta$ -Semialdehyde Dehydrogenase from Infectious Microorganisms

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L-Aspartate- $\beta$ -semialdehyde dehydrogenase (ASA DH) lies at the first branch point in the aspartate metabolic pathway that leads to the formation of the amino acids lysine, isoleucine, methionine, and threonine in most plants, bacteria, and fungi. Since the aspartate pathway is not found in humans, but is necessary for bacterial cell wall biosynthesis, the enzymes in this pathway are potential targets for the development of new antibiotics. The *asd* gene that encodes for ASA DH has been obtained from several infectious organisms and ligated into a pET expression vector. ASA DHs from *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* were expressed as soluble proteins in *Escherichia coli*, while ASA DH from *Helicobacter pylori* was obtained primarily as inclusion bodies. The *V. cholerae* genome contains two *asd* genes. Both enzymes have been expressed and purified, and each displays significant ASA DH activity. The purification of highly active ASA DH from each of these organisms has been achieved for the first time, in greater than 95% purity and high overall yield. Kinetic parameters have been determined for each purified enzyme, and the values have been compared to those of *E. coli* ASA DH. © 2002 Elsevier Science (USA)

L-Aspartate- $\beta$ -semialdehyde dehydrogenase (EC 1.2.1.11; ASA DH) catalyzes the formation of L-aspartate- $\beta$ -semialdehyde (L-ASA) by the reductive dephosphorylation of L- $\beta$ -aspartyl phosphate. ASA DH is the second enzyme in the aspartate metabolic pathway (after aspartokinase) and occupies the first branch

point, making this reaction indispensable for the biosynthesis of one-quarter of the naturally occurring amino acids found in proteins (1). During evolution mammals have lost the enzymes that catalyze these biosynthetic reactions, making the end-product amino acids, L-lysine, L-isoleucine, L-methionine, and L-threonine, essential nutrients. ASA DH activity is also required for biosynthesis of the L-lysine precursor diaminopimelic acid, an integral component of the bacterial cell wall, and dipicolinic acid, important for sporulation in gram-positive bacteria. Disruption of this enzymatic pathway by perturbations of the *asd* gene, which encodes ASA DH, will be lethal to an infecting organism, as demonstrated by work with *Legionella pneumophila* (2), *Salmonella typhimurium* (3), and *Streptococcus mutans* (4).

Site-directed mutagenesis studies, conducted on ASA DH purified from *Escherichia coli*, have identified several important residues that are involved in the catalytic mechanism. These include cysteine-135 as the active site nucleophile (5), an arginine residue (Arg267) that participates in substrate recognition and binding, and a glutamine (Gln162) that assists in catalysis (6). The recently determined structures of the *E. coli* apoenzyme (7) and its complex with NADP and an active-site-directed inactivator (8) reveal an active-site histidine (His274) that facilitates deprotonation of Cys135 to generate the thiolate nucleophile. Sequence homology comparisons show that ASA DH active-site residues are highly conserved among different microorganisms, even when the overall sequence homology is relatively low.

Because of its essential role in amino acid biosynthesis and its relatively well-characterized mechanism, ASA DH is a potentially attractive target for the identification of compounds that can serve as leads for the development of new antibiotics and fungicides. Toward

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this goal, we have purified several ASA DHs from selected infectious organisms for examination in structural and mechanistic studies.

## MATERIALS AND METHODS

**Materials.** Plasmids containing the *asd* genes from *Haemophilus influenzae* KW20, *Vibrio cholerae* El Tor N16961, and *Helicobacter pylori* 26695 from The Institute for Genomic Research were purchased from the American Type Culture Collection. The *asd* gene from *Pseudomonas aeruginosa* PA01 (9) was obtained in a pT7-5 vector with a 1.8-kb *EcoRI/HindIII* insert as a generous gift from Dr. Herbert Schweizer (Colorado State University). Restriction sites were created with the Quickchange Mutagenesis Kit (Stratagene) or by PCR amplification. Restriction enzymes were purchased from New England Biolabs. The pET41a and pET28b vectors, Nova Blue and BL21(DE3) cell lines, and Clonables ligation kits were from Novagen. Plasmid miniprep kits and gel extraction kits were purchased from Qiagen. Tryptone, yeast, and agar were obtained from Difco Laboratories and L-allylglycine was purchased from Sigma. L-ASA was prepared by ozonolysis of L-allylglycine in 1 N HCl according to the method of Black and Wright (10).

**Chromatography and resins.** Enzyme purifications were conducted on an ÄKTA Explorer 100 (Pharmacia) in a chromatography chamber at 4°C. Q Sepharose XL, Procion Red-A, phenyl-Sepharose, and Superdex 200 resins were purchased from Amersham-Pharmacia.  $\omega$ -Aminohexyl-agarose resin was obtained from Sigma.

**Enzyme assay.** Enzyme assays were performed on a Perkin-Elmer Lambda 1 spectrophotometer equipped with a thermostated cell holder connected to a circulating water bath. The assay reaction monitors the oxidative phosphorylation of ASA in the nonphysiological direction in 200 mM Ches, pH 9.0, 40 mM  $KP_i$ , 1 mM NADP at 30°C. Immediately after addition of enzyme, the reaction was initiated by the addition of L-ASA. Measurements were based upon the rate of increasing NADPH absorbance at 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Kinetic parameters for ASA, NADP, and inorganic phosphate were determined by varying the concentration of one substrate at saturating levels of the fixed substrates and fitting the data to an Enzyme Kinetics software package adapted from the programs of Cleland (11). The  $k_{\text{cat}}$  values were calculated based on a subunit molecular weight of 40 kDa. Protein concentrations were determined by the method of Bradford (12).

**Denaturing polyacrylamide gel electrophoresis.** Pre-cast Tris-glycine gels (4% stacking; 10% resolving or 4–20% linear gradient gels) purchased from Bio-Rad were used for SDS-polyacrylamide gel electrophoresis. Visualization was achieved by standard Coomassie

Brilliant Blue R-250 staining for at least 2 h and overnight destaining with a 5% methanol/7% acetic acid solution. To quantitate the relative intensities of the Coomassie blue-stained bands, gel images were scanned into the computer and examined with digitizing software (UN-SCAN, Silk Scientific).

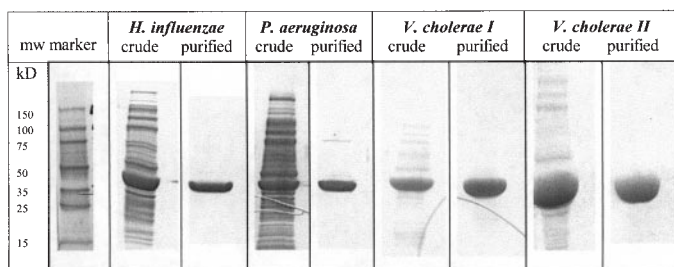
**DNA sequencing.** Gene inserts were first characterized by restriction mapping and then verified with forward and reverse sequencing of the plasmid DNA by the fluorescent dideoxyterminator method using an ABI 3100 capillary sequencer (ACTG, Inc., Northbrook, IL).

## RESULTS

**Purification of *Ha. influenzae* ASA DH** (Swiss-Pro P44801). The *asd* gene from *Ha. influenzae* was removed from pUC18 by digestion with *NdeI* and *EagI* and then ligated into *NdeI/EagI*-digested pET41a using restriction sites created by mutagenesis of the plasmid DNA. A 1- $\mu\text{l}$  aliquot of the ligation reaction was transformed into Nova Blue cells on LB plates containing 30  $\mu\text{g/ml}$  kanamycin sulfate. Positive clones were verified by sequencing. Plasmid DNA for expression was transformed into BL21(DE3) *E. coli* cells. Four liters of LB medium containing kanamycin (30  $\mu\text{g/ml}$ ) was inoculated with 0.1% culture volume and grown until  $A_{600}$  0.6, then induced with 1 mM IPTG, and further shaken at 32°C for 3.5 h. Cell paste was isolated by centrifugation at 12,000g for 20 min. A sample of these cells (3.45 g) was disrupted by sonication in 35 ml of buffer A (50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT) and then centrifuged at 13,000g for 20 min. The supernatant was fractionated by the incremental addition of solid ammonium sulfate. The ASA DH-enriched pellet from the 30 to 60% saturated ammonium sulfate fraction was resuspended in buffer A and dialyzed at 4°C against the same buffer (5  $\times$  2 L). The dialyzed solution was loaded onto a 25-ml Q-Sepharose XL anion-exchange column and eluted with a linear KCl gradient (0 to 1 M) in buffer A. The active fractions, eluted between 0.2 and 0.3 M KCl, were pooled and dialyzed (3  $\times$  2 L) against buffer B (50 mM Hepes, pH 7.0, 1 mM EDTA, 1 mM DTT). The partially purified enzyme was loaded onto a Procion Red-A column and, after being washed with buffer B, was eluted with a 500 mM KCl gradient. The active fractions were pooled, dialyzed against buffer B, and concentrated to 10 mg/ml (Amicon) and aliquots were stored at -20°C. The *Ha. influenzae* ASA DH was determined to be 99% pure by a densitometry scan of a Coomassie blue-stained SDS-polyacrylamide gel (Fig. 1). The results of this purification are summarized in Table 1.

**Purification of *P. aeruginosa* ASA DH** (Swiss-Pro Q51344). The *P. aeruginosa asd* gene was expressed in *E. coli* by ligation of a 1.8-kb *NcoI/HindIII* fragment

A.



B.

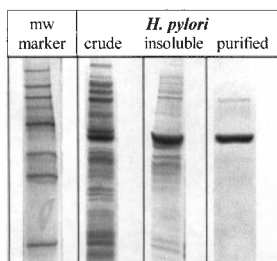


FIG. 1. SDS-PAGE analysis of crude cell extracts and purified samples of ASA DHs from infectious microorganisms. Each lane typically contains at least 10  $\mu$ g of protein sample, except for the overloaded lanes of purified enzymes that could contain up to 100  $\mu$ g of protein to aid in the detection of any low levels of impurities. (A) ASA DHs of *Ha. influenzae*, *P. aeruginosa*, and *V. cholerae* I and II. (B) Crude, insoluble, and purified (Ni-NTA resin) ASA DH of *He. pylori*. Gels were scanned and digitized using UN-SCAN software (Silk Scientific).

containing the *asd* gene into a pET28b vector digested with the same restriction enzymes. The enzyme was expressed in BL21(DE3) *E. coli* cells and grown with 0.5 mM IPTG induction under the conditions described above. Cell paste (3.68 g) was sonicated in 40 ml of buffer A (as above) and the crude mixture was centrifuged at 13,000g for 20 min. The crude extract was loaded onto a 25-ml Q Sepharose XL anion-exchange column and eluted with a linear KCl gradient (0 to 1 M). The active fractions, eluted at approximately 0.3 to 0.4 M KCl, were pooled, and finely ground ammonium sulfate was added to a concentration of ca. 1 M. This mixture was loaded onto a 22-ml phenyl-Sepharose hydrophobic column. The protein was eluted with a decreasing linear gradient of ammonium sulfate (1 to 0 M) in buffer A. Enzyme activity eluted at about 0.4 to 0.3 M salt concentration (conductivity from 80 to 60 mS/cm). The fractions containing homogeneous protein were dialyzed against 10 mM Hepes, pH 7.0, 1 mM EDTA, 1 mM DTT and concentrated to 10 mg/ml. The

purification results are summarized in Table 1. Densitometry scanning of the SDS-PAGE gel showed *P. aeruginosa* ASA DH to be approximately 96% pure (Fig. 1).

**Purification of *V. cholerae* ASA DH I** (Swiss-Pro Q9KQG2). This *V. cholerae asd* gene (GenBank AE004278) was ligated into pET41a as a 1-kb *NdeI/HindIII* fragment. The *asd*-containing plasmid was transformed into BL21(DE3) *E. coli* cells and expressed by induction with 0.75 mM IPTG under the conditions described above. Purification of 6 g wet cell paste was conducted according to the purification previously reported for ASA DH from *E. coli* (13) with minor modifications. Q Sepharose XL anion-exchange resin was substituted for the previously used DEAE-Sephacel (Sigma). The results of this purification are summarized in Table 1. ASA DH from *V. cholerae* was found to be ca. 97% pure by densitometry scanning (Fig. 1).

**Purification of *V. cholerae* ASA DH II** (Swiss-Pro P23247). A second putative *asd* gene from *V. cholerae* (GenBank X55363) was identified, amplified by PCR, ligated into pET41a as a 1-kb *NdeI/EcoRI* fragment, and expressed as described above. A 3.8-g sample (from 2 L cell culture) was sonicated and then centrifugation in 40 ml buffer A (25 mM potassium phosphate, pH 7.0, 1 mM DTT, 1 mM EDTA). The enzyme was loaded onto a Q Sepharose XL column, washed with buffer A, and then eluted with a KCl gradient in buffer A. The pooled fractions, containing about 0.5 M KCl in buffer A, were immediately frozen at  $-20^{\circ}\text{C}$  or, for long-term storage, frozen with 10% glycerol at  $-80^{\circ}\text{C}$ . The results of this purification are summarized in Table 1. This enzyme was estimated to be >97% pure by densitometry scanning (Fig. 1).

TABLE 1

Purification of ASA Dehydrogenases from Infectious Organisms

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
<i>Ha. influenzae</i>				
Crude extract	313	12,000	39	100
Ammonium sulfate	250	19,600	78	163
Q Sepharose XL	102	13,500	132	113
Red-A	55	10,600	192	88
<i>P. aeruginosa</i>				
Crude extract	874	24,100	28	100
Q Sepharose XL	287	21,100	73	88
Phenyl Sepharose	156	20,100	128	83
<i>V. cholerae I</i>				
Crude extract	598	44,300	74	100
Q Sepharose XL	413	39,400	95	89
$\omega$ -Aminohexylagarose	310	30,200	100	68
<i>V. cholerae II</i>				
Crude extract	524	15,600	30	100
Q Sepharose XL	220	18,000	82	115

*Purification of He. pylori ASA DH (Swiss-Pro 025801).* This enzyme was initially expressed as inclusion bodies with an N-terminal hexahistidine tag in pET28b. The soluble fraction of freshly prepared crude extract showed only minimal activity, with a rapid loss of activity observed during the assay. Purification trials on Ni-NTA resin (Qiagen) were conducted under both denaturing (6 M guanidine-HCl) and nondenaturing conditions (elution with increasing imidazole concentrations or decreasing pH). In both cases, soluble and homogeneous protein (ca. 1 mg/ml) of the correct molecular weight was obtained (Fig. 1B), but no ASA DH activity was detected. In another set of experiments, the histidine tag was removed and the *asd* gene was subcloned into pET41 with *NdeI/SalI*. This construct also expressed primarily as inclusion bodies, with a small fraction (2–5%) of soluble enzyme obtained by growing the *E. coli* BL21(DE3) cells at 30°C at 100  $\mu$ M IPTG. Lowering the growth temperature to 20°C while increasing IPTG to 150, 200, or 250  $\mu$ M showed no increase in soluble expression. Under optimized conditions the crude extract displays a specific activity of 11 units/mg; relatively low compared to those values obtained from the other ASA DH extracts. Subsequent dialysis into a variety of buffers at different pH values resulted in precipitation. Attempted purification of the inclusion bodies by washing with BugBuster (Novagen) and solubilizing with Caps, pH 11.0, followed by slow dialysis into Tris or other buffers at a variety of pH values gave soluble and homogeneous protein which displays no catalytic activity.

*Kinetics of ASA dehydrogenases.* The kinetic parameters of the purified enzymes were measured and compared to those previously reported for *E. coli* ASA DH (6). The  $K_m$  values for ASA were found to be the same for each enzyme that was examined. The  $K_m$  values for NADP are virtually identical for the *Ha. influenzae* and *P. aeruginosa* enzymes, while these values are each a factor of 2 higher for both of the *V. cholerae* enzymes compared to *E. coli* ASA DH (Table 2). This, coupled with the lower  $k_{cat}$  values for these enzymes,

gave  $k_{cat}/K_m$  values that are 7- and 21-fold lower than that of *E. coli* ASA DH for *V. cholerae* ASA DH I and II, respectively. The  $K_m$  values of phosphate are also quite comparable for this group of ASA DHs except for *V. cholerae* ASA DH II, which is at least a factor of 4 higher than values for the other ASA DHs studied.

## DISCUSSION

*Expression.* The *E. coli* cell line that was used for expression of these enzymes also contains a functional *asd* gene that could potentially contaminate the recombinant ASA DHs. However, control studies have shown that the amount of *E. coli* ASA DH produced in this cell line under these growth conditions is less than 0.1 unit/mg of protein. These levels are negligible compared with the high expression levels of ASA DH that have been achieved.

While virtually all of the growth conditions screened resulted in good enzyme expression, inclusion body formation was sometimes competitive with soluble enzyme expression when cells were grown at 37°C with 1 mM IPTG. The use of highly enriched media such as Terrific broth or 2 $\times$ YT medium in place of LB medium generally resulted in greater proportions of insoluble enzyme without yielding significantly more total protein. Decreasing the growth temperature of the LB cultures to 32°C and, if necessary, slightly decreasing the IPTG concentration generally led to a reduction of inclusion body formation to acceptable levels. In the case of *He. pylori*, an IPTG concentration of 0.1 mM resulted in a small fraction of soluble enzyme that displayed activity under the assay conditions used in these experiments. However, attempted refolding and recovery of enzyme activity by dialysis into a number of other buffers were unsuccessful in either stabilizing or regenerating enzyme activity.

*Chromatography.* Based on the virtually complete conservation of active site and other essential amino acids identified thus far, we expected these enzymes to show similar affinities for the resins that were used to

**TABLE 2**  
Kinetic Parameters of Purified ASA Dehydrogenases<sup>a</sup>

Organism	$k_{cat}$ (s <sup>-1</sup> )	ASA		Phosphate		NADP	
		$K_m$ (mM)	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )
<i>E. coli</i> <sup>b</sup>	610	0.17	$3.6 \times 10^6$	4.8	$1.3 \times 10^5$	0.19	$3.6 \times 10^6$
<i>Ha. influenzae</i>	330	0.24	$1.4 \times 10^6$	1.6	$2.1 \times 10^5$	0.15	$2.2 \times 10^6$
<i>P. aeruginosa</i>	160	0.12	$1.3 \times 10^6$	1.5	$1.1 \times 10^5$	0.13	$1.6 \times 10^6$
<i>V. cholerae I</i>	120	0.19	$6.3 \times 10^5$	1.1	$1.1 \times 10^5$	0.32	$3.8 \times 10^5$
<i>V. cholerae II</i>	58	0.16	$3.6 \times 10^5$	22	$3.0 \times 10^3$	0.36	$1.6 \times 10^5$

<sup>a</sup> Standard errors on these kinetic parameters are  $\pm 15\%$  or less and were obtained by fitting the data to an Enzyme Kinetics software package adapted from the programs of Cleland (11).

<sup>b</sup> Ouyang and Viola (6).

purify *E. coli* ASA DH. With the exception of *He. pylori* ASA DH, all of the enzymes studied bound well to Q Sepharose XL, a strong anion-exchange resin. However, variations in the binding of each enzyme to dye-ligand-affinity resins and to hydrophobic resins made the choice of a second chromatographic step less straightforward.

*Ha. influenzae* ASA DH. Many nicotinamide-linked dehydrogenases have been shown to bind selectively to dye-ligand resins (14). However, *Ha. influenzae* ASA DH is the only one of the enzymes purified that showed any affinity to the Procion Red-A resin, while displaying no affinity for blue Sepharose or Matrex Green A. Even in this case, the partition coefficient for binding to Red-A was not particularly high. After a brief flowthrough containing mostly impurities, ASA DH slowly began to leech from the resin and had to be quickly eluted by beginning the salt gradient after only minimal wash. Despite this marginal affinity, *Ha. influenzae* ASA DH was obtained as a highly purified sample in excellent overall yield.

*P. aeruginosa* ASA DH. In this case, hydrophobic interaction chromatography was used as the final purification step, with phenyl-Sepharose showing the highest affinity after several hydrophobic resins were tested. Under high-salt (1 M ammonium sulfate) conditions the enzyme bound quite well to the column with a partition coefficient of at least 0.95. However, unlike the other ASA DHs this enzyme has only marginal stability in the assay buffer, losing activity after only 5–10 min.

*V. cholerae* ASA DH I. This ASA DH is the only enzyme that was purified by virtually the same protocol as that reported for *E. coli* ASA DH (13). In this case,  $\omega$ -aminohexylagarose was used successfully as a second anion exchange, displaying a good affinity for the enzyme at low salt concentrations and eluting at roughly 0.4 M KCl. Alternatively, this purification can be effected by binding the enzyme to  $\omega$ -aminohexylagarose under high-salt conditions (1 M ammonium sulfate in phosphate buffer at pH 7.0) and eluting with a decreasing salt gradient. This is in stark contrast to *Ha. influenzae* ASA DH, which has no affinity for this resin under these conditions. Dissociation of *V. cholerae* ASA DH I from the resin occurs at roughly the same conductivity as when an increasing KCl gradient is used. Since going from low- to high-salt conditions gave slightly higher resolution with coelution of fewer impurities, this step was retained as the method of choice.

*V. cholerae* ASA DH II. This construct expressed extremely well, with ASA DH comprising nearly half of the crude extract. The enzyme bound to Q Sepharose XL with a partition coefficient of at least 0.95 and displayed good resolution. These combined factors allowed for a one-step purification in virtually quantitative

yield. Although there was no indication of instability during the purification, it was later observed that the enzyme suffers loss of activity when concentrated, dialyzed, or even stored in the purification buffer at  $-20^{\circ}\text{C}$ . For this reason, kinetic measurements were done without delay after isolation of homogeneous enzyme.

*He. pylori* ASA DH. This enzyme does not bind to cation-exchange resin (HiTrap SP XL) and bound very poorly to both strong (Q Sepharose XL) and weak (DEAE) anion-exchange resins. Attempts at ammonium sulfate fractionation or hydrophobic interaction chromatography were unsuccessful due to precipitation of the protein at even moderately high salt concentrations. The enzyme binds relatively well to blue Sepharose-affinity dye-ligand resin, but suffers severe loss of activity upon elution either with a salt gradient or with NADP. Thus, although relatively homogeneous enzyme has been isolated, samples suitable for crystallization and kinetic trials remain elusive.

*Enzyme stability.* Each of the purified ASA DHs was stored immediately after purification at  $-80^{\circ}\text{C}$  in 10 mM Hepes buffer, pH 7, with 1 mM DTT and 10% glycerol. The enzymes are all relatively stable when stored under these conditions. The *V. cholerae* enzyme II does slowly lose activity even when stored at these conditions and loses activity more rapidly when stored at higher temperatures. Dynamic light scattering experiments have suggested that this loss of activity is related to the increased tendency of this enzyme to aggregate in solution. The *Ha. influenzae* and *E. coli* enzymes are quite stable for several months when stored at  $-20^{\circ}\text{C}$ , and the *V. cholerae* ASA DH I retains nearly 90% of its activity when stored at  $4^{\circ}\text{C}$  over this period of time.

*Catalytic activity.* The kinetic parameters of most of the enzymes studied herein are quite similar to those previously reported for *E. coli* ASA DH. The  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$  values for the *Ha. influenzae* and *P. aeruginosa* enzymes are each within a factor of 3 of the kinetic parameters of the *E. coli* ASA DH. These enzymes, along with the ASA DH I from *V. cholerae*, have 65–70% overall sequence identity with the *E. coli* enzyme. However, the  $k_{\text{cat}}$  of the *V. cholerae* ASA DH I is only 20% that of the *E. coli* enzyme and the  $k_{\text{cat}}/K_{\text{m}}$  for NADP is reduced by a factor of 10. The ASA DH II from *V. cholerae* and the enzyme from *He. pylori* have less than 30% sequence identity with this group of ASA DHs and only 40% identity with each other. The *He. pylori* enzyme is quite unstable under the conditions under which it has been examined, while the  $k_{\text{cat}}$  of ASA DH II from *V. cholerae* is only 10% and the  $k_{\text{cat}}/K_{\text{m}}$  for phosphate and for NADP are less than 5% of the parameters of the *E. coli* enzyme. The lower kinetic parameters of the *V. cholerae* ASA DHs may be compensated for in this organism by having duplicate forms of this enzyme.

These two enzyme forms have less than 25% sequence identity to each other, and it remains to be determined if there are altered structural domains that would allow these ASA DHs to be differentially regulated. Purification of this key enzyme in amino acid biosynthesis from a variety of infectious organisms will allow further kinetic and structural studies that will elucidate the basis of these functional differences.

## ACKNOWLEDGMENTS

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