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A Novel α-Ketoglutarate Reductase Activity of the *serA*-Encoded 3-Phosphoglycerate Dehydrogenase of *Escherichia coli* K-12 and Its Possible Implications for Human 2-Hydroxyglutaric Aciduria

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***Escherichia coli serA*-encoded 3-phosphoglycerate (3PG) dehydrogenase catalyzes the first step of the major phosphorylated pathway of L-serine (Ser) biosynthesis. The SerA enzyme is evolutionarily related to the *pdxB* gene product, 4-phosphoerythronate dehydrogenase, which catalyzes the second step in one branch of pyridoxal 5′-phosphate coenzyme biosynthesis. Both the Ser and pyridoxal 5′-phosphate biosynthetic pathways use the *serC(pdxF)*-encoded transaminase in their next steps. In an analysis of these parallel pathways, we attempted to couple the transaminase and dehydrogenase reactions in the reverse direction. Unexpectedly, we found that the SerA enzyme catalyzes a previously undetected reduction of α-ketoglutarate (αKG) to 2-hydroxyglutaric acid (HGA). Numerous criteria ruled out the possibility that this SerA αKG reductase activity was caused by contamination in the substrate or purified enzyme preparations. HGA was confirmed as the product of the SerA αKG reductase reaction by thin-layer chromatography and by enzyme assays showing that both the D- and L-isomers of HGA were substrates for the reverse (dehydrogenase) reaction. Detailed steady-state kinetic analyses showed that αKG reduction (apparent Michaelis-Menten constant [K_m^{app}] = 88 μM; apparent catalytic constant [k_{cat}^{app}] = 33.3 s^{-1}) and 3-phosphohydroxypyruvate reduction (K_m^{app} = 3.2 μM; k_{cat}^{app} = 27.8 s^{-1}), which is the reverse reaction of 3PG oxidation, were the major in vitro activities of the SerA enzyme. The SerA αKG reductase was inhibited by Ser, D-HGA, 3PG, and glycine (Gly), whereas the D-HGA dehydrogenase was inhibited by Ser, αKG, 3-phosphohydroxypyruvate, and Gly. The implications of these findings for the regulation of Ser biosynthesis, the recycling of NADH, and the enzymology of 2-hydroxyacid dehydrogenases are discussed. Since the same pathway of Ser biosynthesis seems to be present in all organisms, these results suggest that a mutation in the human SerA homolog may contribute to the neurometabolic diseases D- and L-2-hydroxyglutaric aciduria, which lead to the accumulation of D-HGA and L-HGA, respectively.**

serA-encoded 3-phosphoglycerate (3PG) dehydrogenase catalyzes the first committed step in the phosphorylated pathway of Ser biosynthesis in *Escherichia coli* (Fig. 1A) (27, 30, 37). This step is an oxidation of 3PG to 3-phosphohydroxypyruvate (3PHP) with the concomitant reduction of NAD^+ to NADH (27, 37, 38). The SerA enzyme was purified and enzymologically characterized extensively by Pizer and Sugimoto (27, 38, 39). This work showed that the SerA enzyme greatly favors the reverse reaction, which is the reduction of 3PHP to 3PG (27, 38, 39). Therefore, it is likely that the *serC(pdxF)*-encoded 3PHP transaminase plays a critical role in driving Ser biosynthesis forward (Fig. 1A) (27, 38).

Like many enzymes that catalyze the first committed step in a biosynthetic pathway, the SerA 3PG dehydrogenase and 3PHP reductase activities are allosterically inhibited by the end product Ser and by Gly, whose biosynthesis is linked to that of Ser (Fig. 1A) (27, 34a, 37–39). The SerA enzyme was overexpressed and purified by at least two different schemes (34, 38). Recently Schuller, Grant, and Banaszak reported the crystal structure of the SerA tetramer bound to four molecules each of NAD^+ and Ser (34a). This determination was one of the first for an allosteric enzyme that is subject to noncompetitive (V_{max}-type) cooperative inhibition (34a). The SerA structure has several unique features that may play roles in catalysis and allosteric inhibition by Ser and Gly (34a).

SerA 3PG dehydrogenase is a member of a family of evolutionarily related 2-hydroxyacid dehydrogenases that includes the *pdxB*-encoded 4-phosphoerythronate (4PE) dehydrogenase (15, 33, 40, 41). 4PE dehydrogenase catalyzes the second committed step in one branch of pyridoxine 5′-phosphate and pyridoxal 5′-phosphate coenzyme biosynthesis in *E. coli* (20, 25, 33, 46). As in the case of the SerA 3PG dehydrogenase, the forward reaction catalyzed by PdxB 4PE dehydrogenase does not readily occur in vitro. To date, we have been able to detect only a single turnover of 4PE by the purified PdxB enzyme in the forward dehydrogenase direction (25, 46).

The third step in this branch of pyridoxine 5′-phosphate and pyridoxal 5′-phosphate biosynthesis is catalyzed by the same SerC(PdxF) transaminase that catalyzes the second step in the Ser pathway (Fig. 1A) (20, 27, 37). Therefore, we purified the SerA and SerC(PdxF) enzymes as a model to help understand the unusual enzymology of the PdxB 4PE dehydrogenase. We planned to study the coupling of the SerA and SerC(PdxF) reactions in the forward and reverse directions. In trying to couple the reactions in the reverse direction from 3-phosphoserine to 3PG (Fig. 1A), we discovered that the SerA enzyme has an entirely unanticipated activity that reduces α-ketoglutarate (αKG) in the forward direction and oxidizes either D-

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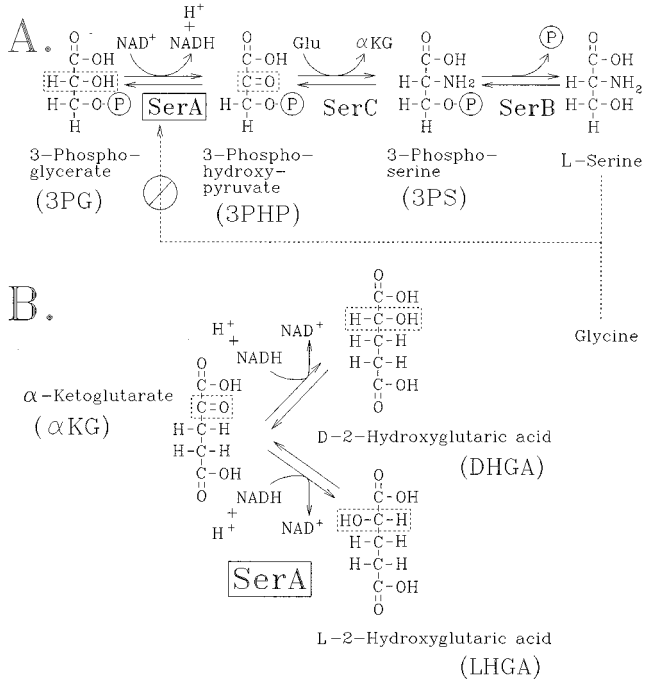

FIG. 1. 3PG dehydrogenase/3PHP reductase and αKG reductase/D- and L-HGA dehydrogenase reactions catalyzed by the *E. coli* SerA enzyme. (A) The phosphorylated pathway of Ser biosynthesis in *E. coli* and other organisms (see the text) (27–29, 37). For simplicity, enzymes are designated by the genes that encode them in *E. coli*, i.e., SerA represents 3PG dehydrogenase/3PHP reductase, SerC(PdxF) represents 3PHP transaminase, and SerB represents 3-phosphoserine phosphatase. Feedback inhibition of SerA activity by Ser and Gly is indicated. See the text for additional details concerning this pathway. (B) SerA-catalyzed αKG reductase, D-HGA dehydrogenase, and L-HGA dehydrogenase activities characterized here. All three activities were inhibited by Ser and Gly (see the text; Table 2; Fig. 3). See the text for additional details.

hydroxyglutaric acid (D-HGA) or L-HGA in the reverse direction (Fig. 1B). In this report, we describe the enzymological characterization of these surprising reactions. We discuss the likely importance of the αKG reductase and D- and L-HGA dehydrogenase activities in the regulation of serine biosynthesis and the recycling of NADH. Our results also suggest a working hypothesis for a contributing cause of the human neurometabolic disease 2-hydroxyglutaric aciduria, which results in the excretion of large amounts of D-HGA or L-HGA in urine and other body fluids (1, 2, 7, 10, 12, 13).

MATERIALS AND METHODS

Materials. αKG (95%), hydroxypyruvate-phosphate-dimethyl ketal, L-HGA (98%), D-HGA (99%), 3PG (95%), oxalacetic (or oxaloacetic) acid (98%), pyruvate (99%), 3-hydroxypyruvate (96%), 2,4-dinitrophenylhydrazine, bromocresol green, Ser, Gly, pyridoxine, NAD^+, and NADH were purchased from Sigma Chemical Co. (St. Louis, Mo.). 3PHP was prepared from hydroxypyruvate-phosphate-dimethyl ketal by following instructions provided by Sigma Chemical Co. Dithiothreitol (DTT) was purchased from United States Biochemical Co. (Cleveland, Ohio) or from Sigma Chemical Co. Hydroxyapatite and molecular mass standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (rabbit muscle phosphorylase B [97.4 kDa], bovine serum albumin [BSA; 66.2 kDa], hen egg white ovalbumin [45 kDa], bovine carbonic anhydrase [31 kDa], soybean trypsin inhibitor [21.5 kDa], and hen egg white lysozyme [14.4 kDa]) were purchased from Bio-Rad (Hercules, Calif.). Glutamate dehydrogenase was purchased from Boehringer Mannheim, Inc. (Indianapolis, Ind.). DEAE-cellulose and 5′-AMP-Sepharose were obtained from Whatman and Pharmacia, respectively.

Bacterial strains and culture media. The following strains of *E. coli* K-12 were used in this study: JM105 [*recA1 endA1 gyrA96 thi hsdR supE44 relA1* Δ(*lac-proAB*)] (New England Biolabs, Inc., Beverly, Mass.), JM105(pSAWT P_{tac}-*serA*$^+$) (34), NU426 [W3110 *sup*(Am) prototroph] (C. Yanofsky collection), TX2683 [NU426 *pdxB*::Km^r(*Hin*dIII) *serA*::Tn*10*dCamMCS *recA1 srl*::Tn*10* (Tet^r)] (46), and TX2675 [NU426 *pdxB*::Km^r(*Hin*dIII) *serA*::Tn*10*dCamMCS] (constructed by generalized transduction of NU402 [NU426 *pdxB*::Km^r (*Hin*dIII)] with P1*vir* phage grown on strain GE1719 [MG1655 *serA*::Tn*10*dCam MCS] [26]).

Luria-Bertani medium (10 g of NaCl per liter) was prepared by dissolving capsules marketed by Bio 101, Inc. (La Jolla, Calif.). Vogel-Bonner (1xE) minimal salts medium containing 0.4% glucose (MMG) was prepared as described previously (8). Ampicillin (100 μg/ml), chloramphenicol (12.5 μg/ml), kanamycin (12.5 μg/ml), or IPTG (isopropyl-β-D-thiogalactopyranoside; 1.5 mM) was added to media as indicated.

Purification of the *E. coli* K12 SerA protein. A 90-ml overnight culture of JM105(pSAWT *serA*$^+$) in Luria-Bertani medium containing ampicillin was inoculated into five 2-liter flasks containing 900 ml each of the same medium. Cultures were grown with vigorous shaking at 37°C. The cultures were allowed to grow to a density of 15 Klett (660 nm) units (≈10^8 CFU/ml) before IPTG was added. The cultures were harvested at 90 to 110 Klett (660 nm) units (≈8×10^8 CFU/ml) by low-speed centrifugation (10 min, 1,000 × *g* at 4°C), washed once with cold 20 mM potassium phosphate (KPO_4) buffer (pH 7.5)–1.0 mM DTT (buffer A), and resuspended in 40 ml of the same buffer. All subsequent steps were performed at 4°C. The collected *E. coli* cells were disrupted by passage through a 20K French pressure cell (Aminco Labs, Inc., Urbana, Ill.) at 20,000 lb/in^2. The resulting suspension was centrifuged at 150,000 × *g* for 60 min (Beckman Ti80 rotor). The supernatant was collected and loaded onto a DEAE-cellulose column (5 by 12 cm) that was equilibrated with buffer A. The column was washed with 600 ml of buffer A and eluted with a linear 0 to 800 mM KCl gradient in buffer A. Eleven 8.3-ml fractions showing high levels of αKG and 3PHP reductase activities (see below) were collected and dialyzed overnight in 50-kDa-cutoff dialysis tubing (Spectrum Medical Industries, Inc., Houston, Tex.) in 5 liters (no changes) of 20 mM KPO_4 (pH 8.2)–1.0 mM DTT (buffer B). The enzyme preparation was applied to a second DEAE-cellulose column (2.5 by 11 cm) equilibrated with buffer B. The column was washed with 100 ml of buffer B and eluted with a linear 0 to 800 mM KCl gradient in buffer B. Ten fractions exhibiting high levels of αKG and 3PHP reductase activities were collected, concentrated to 3 to 5 ml in a medium-size stirred-cell concentrator using a 50-kDa-cutoff membrane (Spectrum Medical Industries, Inc.), and diluted with 20 to 30 ml of 20 mM KPO_4 buffer (pH 7.2)–1 mM DTT (buffer C). Concentration and buffer change to buffer C were repeated two to three more times. The concentrated enzyme preparation (3 to 5 ml) was applied to a hydroxyapatite column (2 by 6 cm) which was equilibrated with buffer C. The column was washed with 90 ml of buffer C and eluted with a linear gradient of 20 to 700 mM KPO_4 buffer (pH 7.2)–1.0 mM DTT. Five fractions showing high levels of reductase activities were pooled, concentrated, and changed to buffer C. At this stage, the SerA enzyme preparation was 95 to 98% pure as judged by SDS-PAGE (5.6% stacking gel; 15% acrylamide gel) (19). Besides the major SerA band with a molecular mass of 46 kDa, only two extremely faint bands, one larger and one smaller than SerA, were apparent in gel lanes containing 5 μg of purified protein stained with Coomassie brilliant blue (data not shown) (47). About 100 mg of SerA enzyme was needed to complete the biochemical and kinetic analyses reported here, because the oxidase reactions were kinetically inefficient (see Table 2). Therefore, the experiments were performed using this hydroxyapatite-purified SerA preparation. Glycerol was added to a final concentration of 15% (wt/vol), and the SerA enzyme preparation was divided into several tubes, which were stored at −75°C and never refrozen before use.

In addition, we purified a sample of hydroxyapatite-purified SerA further by affinity chromatography. We added NaCl (solid) to a final concentration of 0.5 M to two 1.75-mg portions of hydroxyapatite-purified SerA. These samples were loaded onto a 5′-AMP-Sepharose affinity column (0.5 by 6 cm; 1 ml) which was equilibrated with 20 mM KPO_4 buffer (pH 7.2)–1.0 mM DTT–0.5 M NaCl (buffer D). The column was washed with 41 ml of buffer D and eluted with a linear 0 to 12 mM NAD^+ gradient in buffer D. All the fractions eluted with NAD^+ were collected, concentrated, and changed into buffer C (see above) before being stored in 15% glycerol at −75°C. About 2 mg of protein was recovered from the starting 3.5 mg, and no contaminating bands were detected by SDS-PAGE in lanes containing 3.5 μg of protein stained with Coomassie brilliant blue (data not shown). The apparent K_m of αKG [$K_m{}^{app}$(αKG)], the apparent k_{cat} of αKG [$k_{cat}{}^{app}$(αKG)], the $K_m{}^{app}$(3PHP), the $k_{cat}{}^{app}$(3PHP), the ratio of αKG reductase activity to 3PHP reductase activity, and the basic Ser and Gly inhibition patterns of the affinity-purified SerA were the same as those of the hydroxyapatite-purified SerA (Tables 1 and 2) within an experimental error of 10% (data not shown). This result justified the use of the hydroxyapatite-purified SerA in this study and further supported the contention that the αKG reductase and D- and L-HGA oxidase activities were not caused by a contaminant in the SerA enzyme preparations (see Results).

Biochemical and kinetic analyses of the SerA enzyme. Crude extracts for enzyme assays were prepared from the following strains as described previously (47). Strain JM105(pSAWT P_{tac}-*serA*$^+$) was grown with shaking at 37°C in Luria-Bertani medium containing ampicillin to a turbidity of about 20 Klett (660 nm) units (≈2×10^8 CFU/ml). IPTG was added, and the culture was harvested when it reached a turbidity of 60 to 70 Klett units (≈5×10^8 CFU/ml). Strain NU426 (*serA*$^+$ parent) was grown exponentially with shaking at 37°C in MMG containing 1.0 mM Ser and 2.0 μg of pyridoxine per ml and was harvested at a

TABLE 1. Purification of *E. coli* K-12 SerA enzyme[a]

Fraction	Total protein (mg)	αKG reductase sp act (U/mg)	3PHP reductase sp act (U/mg)	αKG reductase total activity (U)	3PHP reductase total activity (U)	Activity ratio (αKG reductase/ 3PHP reductase)	Purification (fold)[b]	Yield (%)
Crude extract	660	3,270	2,800	2.15×10^6	1.84×10^6	1.2	1	100
DEAE-cellulose I (pH 7.5)	116	9,580	8,190	1.11×10^6	0.95×10^6	1.2	3	52
DEAE-cellulose II (pH 8.2)	90	9,700	8,000	0.87×10^6	0.72×10^6	1.2	3	40
Hydroxyapatite	70	11,200	10,000	0.78×10^6	0.70×10^6	1.1	3.5	38
5′-AMP-Sepharose resin[c]	NA	9,700	8,700	NA	NA	1.2	3.0	NA

[a] SerA overexpression, purification, and assays for activities are described in Materials and Methods.

[b] The fold purification values appear low compared with those for the purification schemes of other enzymes (46, 47) because overexpressed SerA amounted to about 30% of the total protein in *E. coli* crude extracts as determined by SDS-PAGE (data not shown). Beyond the DEAE-cellulose I step, the purification mainly removed minor contaminating proteins.

[c] Only 3.5 mg of hydroxyapatite-purified SerA was further purified by AMP-affinity chromatography (recovery ≈2 mg; see Materials and Methods); therefore, total protein, total activities, and yield are not applicable (NA). Experiments described in this report were performed by using the plentiful hydroxyapatite-purified SerA fraction, which was 95 to 98% pure on the basis of SDS-PAGE (data not shown; see Materials and Methods). αKG and 3PHP reductase kinetic parameters and patterns of inhibition by Ser and Gly were the same for hydroxyapatite- and AMP-affinity-purified SerA fractions (Table 2 and data not shown; see Materials and Methods).

turbidity of 60 to 70 Klett units. Strains TX2683 (*serA pdxB recA*) and TX2675 (*serA pdxB*) were grown in the same way as NU426 except that the medium contained chloramphenicol and kanamycin.

The reduction of αKG and 3PHP by SerA was assayed by monitoring the disappearance of NADH, which absorbs at 340 nm (molar extinction coefficient = 6,220 M^{-1} cm^{-1}) (38). Standard reaction mixtures (1 ml each; both substrates saturating) contained 40 mM KPO_4 buffer (pH 7.5), 1.0 mM DTT, 0.25 mM NADH, 5 mM αKG or 0.36 mM 3PHP, and 10 to 300 μg of protein from crude extracts. The decrease in A_{340} was monitored at 37°C for 1 min with a double-beam UV-160 spectrophotometer (Shimadzu Instruments, Inc.). Protein concentrations were determined with a Bradford protein assay kit (Bio-Rad) with BSA as the standard (5). One unit of enzyme activity was defined as the disappearance of 1 nmol of NADH per min at 37°C.

Kinetic analyses of the SerA αKG and 3PHP reductase activities were carried out in reaction mixtures (1 ml each) containing 40 mM KPO_4 buffer (pH 7.5); 1.0 mM DTT; 0.25 mM NADH [which was saturating, since the K_m^{app}(NADH) of the 3PHP reductase is 0.5 μM (38)]; various amounts of αKG or 3PHP; and 5.0 or 0.2 μg of purified SerA protein for the αKG or 3PHP reductase assay, respectively. At pH values higher than 7.5, severe product inhibition and enzyme instability were observed (reference 38 and unpublished results). Initial velocities were measured by monitoring the decrease in A_{340} (disappearance of NADH) at 37°C for 1 min.

Oxidation of 3PG, D-HGA, or L-HGA by SerA was measured by monitoring NADH formation at 37°C (38). Standard reaction mixtures (1 ml; both substrates saturating) contained 40 mM Tris-HCl (pH 8.8); 1.0 mM DTT; 1.0 mM NAD^+; 10.0 mM 3PG or 5.0 mM D-HGA or 10.0 mM L-HGA; and 70.0 or 40.0 or 70.0 μg of purified SerA enzyme for the 3PG, D-HGA, or L-HGA dehydrogenase assay, respectively. Hydrazine (5.0 mM) was added to some 3PG dehydrogenase assay mixtures, because it traps 3PHP and extends the linear range of the assay (38). Control assays showed that hydrazine addition did not affect the D-HGA or L-HGA dehydrogenase assay (data not shown). One unit of enzyme activity was defined as the formation of 1 nmol of NADH per min at 37°C. In kinetic analyses, amounts of 3PG, D-HGA, or L-HGA were varied in the presence of 1.0 mM NAD^+ [which was saturating, since the K_m^{app}(NAD^+) of the 3PG dehydrogenase is 7.8 μM (38)]. Initial velocities were measured by monitoring the increase in A_{340} (NADH formation) at 37°C for 1 min.

The fluorescence of NADH bound to purified SerA protein (excitation wavelength, 340 nm; emission wavelength, 425 nm) was measured in mixtures containing 40 mM KPO_4 buffer (pH 7.5), 1.0 mM DTT, and 75 μg of purified SerA protein at 37°C in a Hitachi F-2000 fluorescence spectrophotometer. The initial fluorescence was about 1,100 arbitrary units (data not shown). Addition of 0.1 mM αKG or 3PHP caused rapid and complete disappearance of fluorescence of bound NADH (emission wavelength, 425 nm). No fluorescence of free NADH (emission wavelength, 465 nm) was detected.

Optimal pH values for the SerA αKG and 3PHP reductase reactions were determined in mixtures (1 ml each) containing 40 mM Tris-HCl (pH varied from 6 to 10), 1.0 mM DTT, 0.25 mM NADH, 5.0 mM αKG or 0.36 mM 3PHP, and 5.0 μg of pure enzyme. The decrease in A_{340} was monitored for 1 min at 37°C. Optimal pH values for the SerA 3PG, D-HGA, and L-HGA dehydrogenase reactions were determined in mixtures (1 ml each) containing 40 mM Tris-HCl

TABLE 2. Steady-state kinetic and inhibition parameters of reactions catalyzed by the SerA enzyme

Reaction[a]	K_m^{app} (mM)	k_{cat}^{app} (s^{-1})[b]	V_{max}^{app} (U/mg)[c]	k_{cat}^{app}/K_m^{app} (s^{-1} M^{-1})	Characteristic(s) of inhibition by Ser[d]	Other type(s) (characteristics) of inhibition[e]
3PG oxidation	1.2	0.55	183	5.0×10^2	Uncompetitive[f]; K_i = 3.8 μM[f]; I_{50} = 44 μM	Glycine
3PHP reduction	0.0032	27.8	9,270	8.7×10^6	Noncompetitive[f]; I_{50} = 12.5 μM	Glycine
αKG reduction	0.088	33.3	11,100	3.8×10^5	Noncompetitive; K_i = 5.4 μM; I_{50} = 7.6 μM	Competitive by D-HGA (K_i = 0.37 mM[g]); noncompetitive by 3PG (K_i = 12.7 mM); glycine
D-HGA oxidation	0.37	0.71	237	1.9×10^3	Uncompetitive; I_{50} = 36 μM	Competitive by αKG (K_i = 30 μM[g]); competitive by 3PHP (K_i = 29 μM); glycine
L-HGA oxidation	2.9	0.25	83.3	9.0×10^1	I_{50} = 175 μM	Glycine

[a] Steady-state kinetic analyses were performed as described in Materials and Methods and (38, 46, 47). No deviations from linearity were observed on plots of kinetic data. Standard errors of parameters were less than 10%.

[b] k_{cat}^{app} values were calculated by assuming that 100% of the purified SerA protein was active. No difference in αKG and 3PHP reductase kinetic parameters between hydroxyapatite-purified SerA (95 to 98% pure) and AMP-affinity-purified SerA (electrophoretically pure) (Table 1; see Materials and Methods) was noted.

[c] V_{max}^{app}, apparent V_{max}.

[d] I_{50} is the concentration of Ser that gave 50% inhibition in the standard enzyme assays with both substrates saturating (Fig. 3; see Materials and Methods). The kinetic limitations (relatively high K_m^{app} and low k_{cat}^{app}) of the SerA D-HGA and L-HGA dehydrogenase reactions precluded detailed inhibition studies, and only I_{50} values were determined.

[e] Concentrations of Gly much higher than those of Ser were required for inhibition, as noted previously for the 3PG dehydrogenase/3PHP reductase reactions (38). The following levels of Ser and Gly inhibition were measured in standard assays (see Materials and Methods): for αKG reductase, 61% by 10 μM Ser and 40% by 1,000 μM Gly; for 3PHP reductase, 56% by 10 μM Ser and 32% by 1,000 μM Gly; for 3PG dehydrogenase, 67% by 100 μM Ser and 50% by 3,000 μM Gly; for D-HGA dehydrogenase, 62% by 100 μM Ser and 45% by 3,000 μM Gly; for L-HGA dehydrogenase (15 mM L-HGA), 16% by 100 μM Ser and 8% by 10,000 μM Gly; and for oxaloacetate reductase (10 mM oxaloacetate), 55% by 10 μM Ser and 45% by 1,000 μM Gly.

[f] Inhibition type or value taken from reference 38.

[g] For product inhibition.

(pH varied from 6 to 10); 1.0 mM DTT; 1.0 mM NAD^+; 70 μg of purified SerA; and 10 mM 3PG plus 5.0 mM hydrazine, 5.0 mM D-HGA alone, or 10.0 mM L-HGA alone. The increase in A_{340} was monitored for 1 min at 37°C.

Optimal temperatures of the SerA αKG and 3PHP reductase reactions were determined in mixtures (1 ml each) containing 40 mM KPO_4 buffer (pH 7.5), 1.0 mM DTT, 0.25 mM NADH, 5.0 mM αKG or 0.36 mM 3PHP, and 5.0 μg of purified SerA. The decrease in A_{340} was determined for 1 min at temperatures between 25 to 60°C. Thermal stabilities of the SerA αKG and 3PHP reductase activities were determined by heating 100 μg of purified SerA in 40 mM KPO_4 buffer (pH 7.5)–1.0 mM DTT at various temperatures for 3 min. Heated samples were rapidly chilled on ice, and enzyme assays were carried out in reaction mixtures (1 ml each) containing 40 mM KPO_4 (pH 7.5), 1.0 mM DTT, 0.25 mM NADH, 5.0 mM αKG or 0.36 mM 3PHP, and 3.5 μg of the heated enzyme. The decrease in A_{340} was measured at 37°C for 1 min for each sample.

The specificity of the SerA reductase reaction for substrates other than αKG and 3PHP was determined by monitoring NADH disappearance (i.e., decrease in A_{340}) at 37°C for 1 min in reaction mixtures (1 ml each) containing 40.0 mM Tris-HCl (pH 8.8); 1.0 mM DTT; 0.25 mM NADH; 5.0 μg of purified SerA; and 10 mM oxaloacetate, pyruvate, or 3-hydroxypyruvate.

Product analyses of the SerA αKG reductase and D-HGA dehydrogenase reactions. SerA αKG reaction mixtures (3 ml each) containing 20 mM KPO_4 buffer (pH 7.5), 1.0 mM DTT, 5.0 mM αKG, 5.5 mM NADH, and 1.1 mg of pure SerA enzyme or control reaction mixtures containing 1.1 mg of BSA instead of SerA were incubated at 37°C for 55 min. The disappearance of NADH was monitored as a decrease in A_{340}. No decrease in A_{340} was detected in the BSA control reaction mixtures. Activated charcoal (10% [wt/vol]) was added to the reaction mixtures, and charcoal extractions of NADH and NAD^+ were carried out with gentle shaking at room temperature for 1 h. Charcoal was removed by centrifugation (10 min, 2,500 × *g*) and then by passage of clear supernatants through 0.45-μm-pore-size syringe filters. The extracted reaction products were analyzed by thin-layer chromatography on Whatman PE Sil G-silica plates (250-μm layer; 20 by 20 cm). Plates were developed in freshly mixed butanol-formic acid-water (8:3:2) at room temperature, air dried, and sprayed with bromocresol green (0.04% [wt/vol] in 95% [wt/vol] ethanol) or 2.4-dinitrophenylhydrazine (0.4% [wt/vol] in 2.0 M HCl) (11).

SerA D-HGA dehydrogenase reaction mixtures (3 ml each) containing 20.0 mM KPO_4 buffer (pH 8.2), 1.0 mM DTT, 5.0 mM D-HGA, 5.0 mM NAD^+, and 0.5 mg of purified SerA enzyme or BSA (control) were incubated at 37°C for 50 min. Reaction mixtures were additionally incubated at 65°C for 15 min and then mixed with 10% (wt/vol) activated charcoal and extracted as described above. αKG was detected in the extracted products by its use as a substrate by glutamate dehydrogenase, which reduces αKG to glutamate and concomitantly oxidizes NADH to NAD^+ (9). Glutamate dehydrogenase reaction mixtures contained 0.45 ml of the extracted product from D-HGA dehydrogenase reactions, 0.03 ml of 0.5 M ammonium acetate, 0.0125 ml of 10 mM NADH, and 0.015 ml of glutamate dehydrogenase at 5.0 mg/ml (9). The disappearance of NADH was monitored as a decrease in A_{340} with time at 37°C.

RESULTS

Discovery and evidence that the SerA α-KG reductase activity is not a contaminating activity. We overexpressed SerA 3PG dehydrogenase from strain JM105 containing the pSAWT (P_{tac}-*serA*$^+$) expression vector (34) and purified the SerA enzyme by the scheme outlined in Table 1 and Materials and Methods. We also purified His_6-SerC(PdxF) 3PHP transaminase as described before (46). We attempted to couple the SerC(PdxF) and SerA reactions in the reverse direction by first mixing αKG, NADH, and the purified SerA and His_6-SerC(PdxF) enzymes (Fig. 1A) in the cuvette of a fluorescence spectrophotometer at 37°C. However, before we added the 3PS substrate, we observed rapid oxidation of NADH as indicated by an immediate drop in the fluorescence intensity (excitation wavelength, 340 nm; emission wavelength, 465 nm). By testing combinations of components of the reaction mixture, we found that the SerA enzyme seemed to possess a strong αKG reductase activity (Fig. 1B). An early study of the coupled reverse reactions of *E. coli* SerC(PdxF) and the chicken liver homolog of SerA may not have detected this αKG reductase activity because reactions were started by addition of αKG to mixtures already containing 3PS (17a) instead of by 3PS addition as in our attempt.

Several lines of evidence ruled out contamination of the αKG substrate or purified SerA enzyme as a source of this novel αKG reductase activity. In these and most subsequent experiments, we measured oxidation of NADH to NAD^+ by a decrease in A_{340} (Materials and Methods). In 1.0-ml enzyme reaction mixtures containing 0.25 mM NADH and 0.13 mM or 0.25 mM αKG, the A_{340} dropped from 1.5 to 0.69 or 0.089, respectively, in 15 min at 37°C. This drop in A_{340} indicated that 0.13 or 0.23 mM NADH was consumed, which was equivalent to the total initial amounts of αKG in the reaction mixtures. Thus, it is unlikely that some contaminant in the αKG was acting as a substrate, since the purity of the αKG was at least 95% (see Materials and Methods). This conclusion was confirmed by thin-layer chromatographic analyses of substrates and products (see below).

A minor contaminating enzyme in the SerA preparation also could not account for the αKG reductase activity. The ratio of the αKG reductase activity to the 3PHP reductase activity remained constant at 1.2 through each step of the SerA enzyme purification (Table 1), and the elution profiles for the two activities were superimposable (data not shown). The αKG and 3PHP reductase activities were also correlated with the *serA* genotype. We found the following αKG and 3PHP reductase specific activities, respectively, in crude extracts of strains grown as described in Materials and Methods: JM105 (pSAWT) (SerA overexpressor from a *serA*$^+$ minimal clone), 3,266 and 2,796 U/mg (Table 1); NU426 (wild-type *serA*$^+$ strain), 114 and 97 U/mg; TX2683 (*serA pdxB recA*), 3.5 and 3.6 U/mg, and TX2675 (*serA pdxB*), 4.1 and 3.9 U/mg. Again, the ratio of αKG reductase activity to 3PHP reductase activity was 1.2 in extracts of NU426. Ser (0.025 mM) inhibited 88% of the αKG and 3PHP reductase activities in the crude extract of NU426 but did not inhibit the residual activities observed in the crude extracts of *serA* mutants TX2683 and TX2675 (data not shown). The low-level (≈3%) residual activity in extracts of the *E. coli serA* mutant strains may be due to substrate ambiguity of another dehydrogenase.

In addition, the αKG reductase activity of the purified SerA enzyme was inhibited by the allosteric inhibitors, Ser and Gly; by the competing substrate 3PG; and by the product, D-HGA (Fig. 1; Table 2). Finally, αKG reduction was accompanied by the oxidation of NADH molecules that were bound to the purified SerA enzyme. Previously, it was shown that the purified SerA enzyme contains bound NADH molecules that can be detected by a fluorescence emission at 425 nm (excitation wavelength, 340 nm), which is different from that of free NADH at 465 nm (39). When 3PHP was added to the purified enzyme in the absence of additional NADH, this fluorescence at 425 nm was rapidly lost (39). Likewise, our purified SerA preparation fluoresced at 425 nm, and this fluorescence was rapidly and completely lost upon the addition of either 3PHP or αKG. This is an important control against contamination, because a minor contaminating activity would not cause the complete loss of the SerA-NADH fluorescence at 425 nm. Taken together, these results show that the SerA enzyme is both a 3PHP and an αKG reductase.

HGA is the product of the SerA αKG reductase reaction. Two lines of evidence support the conclusion that HGA is the product of the αKG reductase reaction (Fig. 1B). First, we analyzed reactants and products by thin-layer chromatography (see Materials and Methods). We allowed the SerA αKG reaction to go to completion and removed NADH and NAD^+ by charcoal extractions (see Materials and Methods). Control reaction mixtures containing αKG, NADH, and BSA, instead of SerA, gave a single spot at the same position ($R_f \approx 0.61$) as did the pure αKG standard when reacted with the aldehyde and ketone detection reagent, 2,4-dinitrophenylhydrazine (11). In contrast, the product of reaction mixtures containing αKG, NADH, and SerA protein was not detectable with 2,4-dinitro-

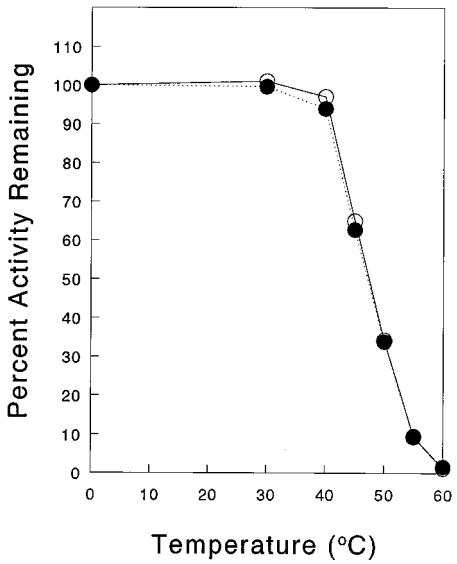

FIG. 2. Heat inactivation of SerA αKG and 3PHP reductase activities. Enzyme preparations were incubated at the indicated temperatures for 3 min and kept on ice until assayed (see Materials and Methods). One hundred percent activity corresponded to unheated samples (αKG reductase, 9,714 U/mg; 3PHP reductase 7,486 U/mg). Open circles, αKG reductase; closed circles, 3PHP reductase.

phenylhydrazine (data not shown). Thus, the ketone group of αKG was reduced by the SerA enzyme. Mobility markers showed that we were unable to resolve pure samples of αKG, D-HGA, and L-HGA ($R_f \approx 0.61$), which were detected by the carboxylic acid reagent, bromocresol green (11). Nonetheless, the substrates and products of the completed αKG reaction showed spots ($R_f \approx 0.61$) when reacted with bromocresol green. This is the expected result, because αKG, D-HGA, and L-HGA all contain carboxylic acid groups (Fig. 1B).

Second, we showed that D-HGA and to a lesser extent L-HGA act as substrates for the SerA enzyme in the reverse (dehydrogenase) direction (Fig. 1B). We performed detailed kinetic analyses of the D- and L-HGA dehydrogenase reactions (see below) and showed that oxidation of D-HGA by SerA leads to the formation of αKG. In the latter experiments, we detected αKG by its use as the substrate for glutamate dehydrogenase in a subsequent reaction (data not shown; see Materials and Methods). We were unable to detect the formation of αKG from L-HGA because the SerA L-HGA dehydrogenase activity was probably strongly product inhibited by αKG (see below). Taken together, these data strongly suggest that HGA is the product of the SerA αKG reductase reaction, although it was not possible to tell whether the D- or the L-isomer of HGA was formed.

Biochemical and kinetic properties of the SerA αKG reductase and D-HGA and L-HGA dehydrogenase activities. SerA protein purified as outlined in Table 1 was stable for weeks at 4°C without significant loss of activity and for months at −75°C in 15% glycerol (data not shown). Plots of SerA αKG and 3PHP reductase activities from 1-min incubations at different temperatures (see Materials and Methods) were identical and bell shaped with an optimum of 45 to 50°C at pH 7.5. However, the purified SerA enzyme was relatively thermally unstable (Fig. 2). Incubation of the enzyme at 45°C for 3 min caused a 40% drop in both αKG and 3PHP reductase activities (Fig. 2). It is noteworthy that the thermal stability curves for the αKG and 3PHP activities were superimposable (Fig. 2). Likewise, the SerA αKG and 3PHP reductase activities had identical pH profiles with an optimum of 8.5 at 37°C (data not shown; see Materials and Methods). The levels of the two activities rose steeply with increasing pH from 6 to 8.5 and then dropped sharply as the pH increased further. These findings again indicate that the αKG and 3PHP reductase activities are catalyzed by the same protein. The L-HGA, D-HGA, and 3PG dehydrogenase activities of the purified SerA enzyme also exhibited similar pH profiles at 37°C with optimal pH values of 9.0 for D- and L-HGA and 9.5 for 3PG (data not shown). The difference in pH optima for the reductase and dehydrogenase activities may reflect the complicated SerA catalytic mechanism, which includes isomerization steps and possibly protein conformational changes (34a).

We performed steady-state kinetics analyses of the SerA enzyme for its five substrates (Table 2). The K_m^{app} values obtained for 3PG and 3PHP agreed closely with those determined previously by Sugimoto and Pizer (38). In terms of the apparent substrate affinities (K_m^{app}), apparent turnover numbers (k_{cat}^{app}), and substrate specificities (k_{cat}^{app}/K_m^{app}), these analyses showed that the two major activities of the SerA enzyme are 3PHP and αKG reduction, rather than 3PG oxidation in Ser biosynthesis (Fig. 1B; see the introduction).

All five SerA activities were inhibited by Ser (Table 2) and Gly (Table 2 and data not shown). As expected from previous work (38), much highter concentrations of Gly than of Ser were required for inhibition of all five SerA activities (Table 2 and data not shown). The SerA αKG reductase activity was competitively product inhibited by D-HGA, and the D-HGA dehydrogenase activity was competitively product inhibited by αKG (Table 2). It was noteworthy that 3PG noncompetitively inhibited the αKG reductase activity and 3PHP competitively inhibited the D-HGA dehydrogenase activity (Table 2). This finding again supports the contention that the αKG reductase and D-HGA dehydrogenase activities are catalyzed by the SerA enzyme.

L-HGA was a relatively poor substrate for the SerA enzyme (Table 2). This observation raised the possibility that the apparent L-HGA activity was due to contamination of the commercially available L-HGA by D-HGA. However, this hypothesis was not supported by further investigations of SerA inhibition by Ser (Fig. 3). Inhibition of the D-HGA and 3PG dehydrogenase (Fig. 3A) and αKG and 3PHP reductase (Fig. 3B) activities by Ser was negatively cooperative. In contrast, Ser inhibition of the L-HGA activity was linear (Fig. 3C) and distinct from that of the D-HGA reaction (Fig. 3A). The curves for the inhibition of the D-HGA and 3PG dehydrogenase activities by Ser were indistinguishable (Fig. 3A). Likewise, the Ser inhibition curves for the αKG and 3PHP reductase activities had similar shapes (Fig. 3B). Finally, we tested whether other compounds structurally related to αKG served as substrates of the SerA enzyme. The four-carbon analog oxaloacetate was weakly reduced by the SerA enzyme (≈5% of the αKG and 3PHP reductase specific activities), whereas the three-carbon compounds pyruvate and 3-hydroxypyruvate, which lack two negatively charged groups, were not detectably reduced (data not shown). Thus, the three-carbon carboxylic acid-phosphoric ester 3PHP and the five-carbon dicarboxylic acid αKG were kinetically the best substrates of the SerA enzyme.

DISCUSSION

We found that *E. coli serA*-encoded 3PG dehydrogenase/3PHP reductase (Fig. 1A) possesses a novel αKG reductase/D- and L-HGA dehydrogenase activity (Fig. 1B; Table 2). Numer-

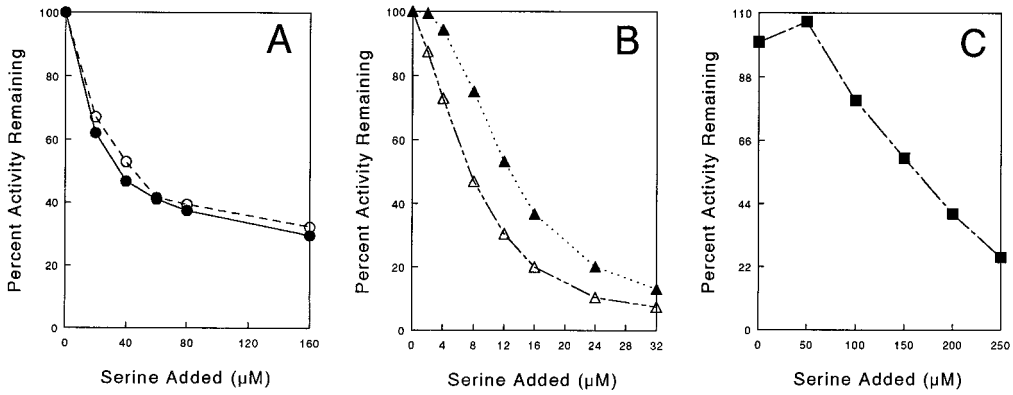

FIG. 3. Serine inhibition of SerA 3PG, D-HGA, and L-HGA dehydrogenase and αKG and 3PHP reductase activities. (A) D-HGA dehydrogenase (closed circles) and 3PG dehydrogenase (open circles) activities. Standard assay mixtures at saturating substrate concentrations contained 50 μg of purified SerA enzyme and no hydrazine (see Materials and Methods). One hundred percent activity corresponded to samples without added Ser (D-HGA dehydrogenase, 227 U/mg; 3PG dehydrogenase, 150 U/mg). (B) αKG reductase (open triangles) and 3PHP reductase (closed triangles) activities. Standard assays at saturating substrate concentrations contained 5.0 μg of purified SerA enzyme (see Materials and Methods). One hundred percent activity corresponded to samples without added Ser (αKG reductase, 8,840 U/mg; 3PHP reductase, 6,680 U/mg). (C) L-HGA dehydrogenase activities. Standard assay mixtures at saturating substrate concentration contained 70 μg of purified SerA enzyme and no hydrazine (see Materials and Methods). One hundred percent activity corresponded to samples without added Ser (L-HGA dehydrogenase, 73 U/mg).

ous lines of evidence, including copurification (Table 1); enzyme levels in overexpressor and mutant strains (see Results); oxidation of bound NADH (see Results); thermal inactivation (Fig. 2); and kinetic patterns of inhibition by Ser, Gly, substrates, and products (see Results; Table 2; Fig. 3) ruled out the possibility that this novel αKG reductase/D- and L-HGA dehydrogenase activity was due to a contaminant in our purified SerA enzyme preparations. In fact, our results showed that 3PHP reduction and αKG reduction are kinetically the major in vitro activities of the SerA enzyme (Fig. 1; Table 2) (38). Finally, we showed that HGA is the product of the SerA αKG reductase reaction (Table 2; see Results). The SerA enzyme used both the D- and L-isomers of HGA as substrates in the dehydrogenase direction, although D-HGA was greatly preferred (Table 2; Fig. 3).

These findings have several important implications for cellular metabolism and human disease. The D- and L-isomers of HGA were previously detected in several organisms, including bacteria and humans, but the pathways leading to D-HGA and L-HGA synthesis were unknown (18, 32). This report represents the first time that a route of HGA biosynthesis has been demonstrated. Clinical and biochemical studies established a correlation between elevated levels of D-HGA and L-HGA in the urine, cerebrospinal fluid, and plasma of patients and the neurometabolic diseases D- and L-2-hydroxyglutaric aciduria, respectively (1, 7, 10, 12, 13). Yet the precise metabolic block or disorder that leads to the accumulation of D-HGA or L-HGA in these patients is unknown. It was suggested that a defect in an enzyme involved in the metabolism of D-HGA or L-HGA might underlie this metabolic disorder (2, 7). Recently, a low level of L-HGA dehydrogenase activity was detected in normal human and rat tissues (2).

Our results suggest that mutations in the human homolog of the *E. coli* SerA enzyme may contribute to the accumulation of D-HGA or L-HGA characteristic of human D- or L-2-hydroxyglutaric aciduria. The phosphorylated pathway of serine biosynthesis (Fig. 1A) has been found in cultured human cells (28, 29, 31), chicken liver (16, 17, 42, 43), and mouse cell extracts (6) and may be present in all organisms. The purified chicken liver homolog and the *E. coli* SerA protein share fundamental enzymological properties, including size (homotetramers with a molecular mass of about 165 kDa and subunit molecular masses of 40 to 43 kDa [16, 43]) and kinetic properties (high apparent affinities [i.e., low K_m^{app} values] for NADH and 3PHP compared with 3PG and NAD^+ [Table 2] [42] and 3PHP reduction kinetically favored over 3PG oxidation [42]). Likely 3PG dehydrogenase homologs from mouse and *E. coli* SerA show a significant number of amino acid identities (33%) and similarities (54%) over their nucleotide- and substrate-binding domains (GenBank accession number L21027) (22a, 34a). Interestingly, the K_m^{app} and pH optimum of the L-HGA dehydrogenase activity detected in rat crude extracts were estimated to be 5 to 10 mM and 8.7, respectively (2), and these values match those for the *E. coli* SerA enzyme (2.9 mM [Table 2] and pH 9.0 [see Results]). These similarities suggest that the human SerA homolog may also possess an αKG reductase activity that forms D-HGA and L-HGA. This hypothesis is certainly testable.

Our results also have important implications for the regulation of Ser biosynthesis in *E. coli* and perhaps other organisms (see above). αKG is a central metabolite of the tricarboxylic acid cycle and plays an important role in amino acid metabolism (3, 14, 24). The total in vivo concentration of αKG in *E. coli* was estimated to be 0.45 mM (21, 35), which far exceeds the K_m^{app} (0.088 mM [Table 2]) of the SerA enzyme for αKG. The total in vivo concentration of 3PG is about 0.75 to 1.3 mM (22, 23), which approximates the K_m^{app} (1.2 mM [Table 2]) of the SerA enzyme for 3PG. By using the equilibrium constant for the 3PG dehydrogenase forward reaction (7×10^{-11} M [38]) and the total cellular concentrations of NAD^+ (0.016 mM) and NADH (0.79 mM) (4) and 3PG (see above), we can roughly estimate the cellular 3PHP concentration to be 0.003 mM, which is very close to the K_m^{app} of the SerA enzyme for 3PHP (Table 2). One implication of these concentrations and the SerA kinetic parameters (Table 2) is that Ser biosynthesis probably must compete with αKG reduction in vivo. Therefore, the cellular αKG concentration may regulate Ser biosynthesis and one-carbon metabolism directly by modulating the SerA 3PG dehydrogenase activity. Consistent with this idea, we found that anaerobic growth of *E. coli* was inhibited on MMG plates containing 1% (wt/vol) αKG at 37°C, and this inhibition was reversed by 0.42 mM Ser (48). Both the SerA 3PG dehydrogenase and SerA αKG reductase activities are inhibited by Ser at about the same K_i (Table 2) and by Gly. Consequently,

both Ser biosynthesis and αKG reduction respond to cellular Ser and Gly supply. Although 3PG noncompetitively inhibited αKG reductase activity, the relatively high K_i (12.7 mM [Table 2]) suggests that this inhibition may not be physiologically significant.

Besides its possible role in regulating Ser biosynthesis, the SerA αKG reductase activity may play a role in recycling NADH back to NAD^+, especially during anaerobiosis. NADH generated by glyceraldehyde 3-phosphate dehydrogenase must be oxidized back to NAD^+ to sustain glycolysis under anaerobic conditions (14). As much as 15% of the carbon atoms derived from glucose are used in the biosynthesis of Ser, which feeds into glycine biosynthesis and one-carbon metabolism (30). A considerable amount of cellular NAD^+ (6 molecules for every 10 glucose molecules) is converted to NADH during the synthesis of the Ser precursors, 3PG and 3PHP (Fig. 1A) (30). The SerA αKG reductase activity may act to recycle some of this NADH and to link Ser supply and biosynthesis directly to NAD^+ supply. The metabolic fates of D-HGA and L-HGA are unknown, although these compounds have been detected in *E. coli* (32) as well as in mammalian tissues and human patients with 2-hydroxyglutaric aciduria (see above).

As suggested by its crystal structure, the SerA enzyme likely uses overlapping active sites for 3PG oxidation/3PHP reduction and αKG reduction/D- and L-HGA oxidation. An indication that these sites may overlap is the competitive inhibition of D-HGA oxidation by 3PHP (Table 2). The COOH-HCOH groups of 3PG and D- and L-HGA and the COOH-CO groups of 3PHP and αKG (Fig. 1) are likely positioned for hydride transfer with the NAD ring by a conserved histidine (position 292)-glutamate (position 269) pair and an arginine (position 240) residue in the SerA active site (34a). The phosphoric ester group of the three-carbon 3PG/3PHP substrates and the C(5)OOH group of the five-carbon αKG/D- and L-HGA substrates probably make contacts in an anionic-binding pocket near the serine residue (position 61) in the SerA active site (34a). Presumably, relatively poor four-carbon substrates, such as oxaloacetate (5% activity; see Results), do not make suitable contacts within this anionic-binding pocket to allow efficient catalysis.

Finally, our findings address a long-standing issue concerning the stereochemistry of NAD^+ reduction by the SerA enzyme. *E. coli* SerA enzyme is a member of a D-isomer-specific family of 2-hydroxyacid dehydrogenases which includes *E. coli* PdxB 4-phosphoerythronate dehydrogenase; D-lactate dehydrogenases from *Lactobacillus plantarum* and *Lactobacillus casei*; *Pseudomonas* formate dehydrogenase; and NADH-dependent hydroxypyruvate reductase, glycerate dehydrogenase, and 2-hydroxyisocarproate dehydrogenase from cucumber (15, 33, 40, 41). In this family, only the SerA and PdxB enzymes use phosphorylated substrates. It has been a puzzle why *E. coli* SerA and its homolog from peas transfer hydride ions to the A side of the nicotinamide ring of NAD^+ during 3PG oxidation (Fig. 1A) instead of to the B side as do all other dehydrogenases that use phosphorylated substrates (36, 44, 45). Our finding that the SerA enzyme also uses the nonphosphorylated substrates αKG and D- and L-HGA and to a small extent (≈5%) oxaloacetate (see Results) helps to resolve this puzzle. The stereospecificity of hydride transfer by dehydrogenases/reductases that use nonphosphorylated substrates occurs either to the A side or to the B side of the nicotinamide ring of NAD^+ (45). Therefore, the evolutionary ancestor of the SerA enzyme may have first carried out αKG reduction and D- and L-HGA oxidation to the A side of the nicotinamide and then later acquired the 3PG oxidation and 3PHP reduction activities after the stereospecificity of hydride transfer was fixed. This hypothesis can partly be tested by determining whether the PdxB 4PE dehydrogenase, which is thought to have evolved from the SerA enzyme (33), uses unorthodox hydride transfer to the A side of the nicotinamide ring of NAD^+. It will also be interesting to learn whether the PdxB 4PE dehydrogenase, like its SerA homolog, possesses an αKG reductase activity.

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