Substrate Specificity and Identification of Functional Groups of Homoserine Kinase from *Escherichia coli*†

Xuewen Huo and Ronald E. Viola*

Department of Chemistry, The University of Akron, Akron, Ohio 44325-3601

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ABSTRACT: Homoserine kinase, an enzyme in the aspartate pathway of amino acid biosynthesis in *Escherichia coli*, catalyzes the conversion of L-homoserine to L-homoserine phosphate. This enzyme has been found to have broad substrate specificity, including the phosphorylation of L-homoserine analogs where the carboxyl functional group at the α-position has been replaced by an ester or by a hydroxymethyl group. Previous pH profile studies [Huo, X., & Viola, R. E. (1996) *Arch. Biochem. Biophys. 330*, 373−379] and chemical modification studies have suggested the involvement of histidinyl, lysyl, and argininyl residues in the catalytic activity of the enzyme. With the assistance of sequence alignments, several potential amino acids have been targeted for examination. Site-directed mutagenesis studies have confirmed a role for arginine-234 in the binding of the carboxyl group of L-homoserine, and the involvement of two histidines at the homoserine binding site. Mutations at these sites have led to the decoupling of the kinase activity from an inherent ATPase activity in the enzyme, and suggest the presence of independent domains for the binding of each substrate in homoserine kinase.

Homoserine kinase (EC 2.7.1.39), the fourth enzyme in the aspartate pathway of amino acid biosynthesis in microorganisms, catalyzes the phosphorylation of L-homoserine to L-homoserine phosphate, an intermediate in the production of L-threonine, L-methionine, and L-isoleucine (Cohen, 1983). The enzyme is a homodimer with a subunit molecular mass of 33 000 daltons, and the thrB gene coding for the enzyme in *Escherichia coli* has been sequenced (Cossart et al., 1981). This gene has been cloned, and the enzyme has been overexpressed and purified to homogeneity (Huo & Viola, 1996). Several analogs of L-homoserine have previously been identified as alternative substrates for homoserine kinase (Shames & Wedler, 1984), and kinetic studies have suggested that there are two L-homoserine binding sites, one catalytic and the other inhibitory. An examination of the pH dependence of the kinetic parameters has revealed two cationic groups with p*K* values near 8.0 that must be in the correct protonation state for optimal catalytic activity. Chemical modification with group-specific reagents has indicated the involvement of a histidinyl and a lysyl residue at or near the enzyme active site (Huo & Viola, 1996).

A series of structural analogs of homoserine have been synthesized, and these analogs have been used to extensively examine the substrate specificity of the enzyme. Additional chemical modification and sequence homology studies have been used to identify important amino acid residues in the enzyme as potential mutagenic targets. Replacement of these functional groups by site-directed mutagenesis has led to significant alterations in the substrate specificity and in the catalytic activity of homoserine kinase, and thus has allowed a subsequent assignment of functional roles to these amino acids.

EXPERIMENTAL PROCEDURES

Materials. All purchased chemicals were reagent grade and were used without further purification. Double-stranded M13mp18 and M13mp19 replication form DNA were purchased from New England Biolab. (*p*-Hydroxyphenyl)-glyoxal (HPG)[1] was obtained from Pierce. Kits for plasmid DNA and single-stranded DNA purification and for DNA extraction from agarose gel were purchased from QIAGEN. The native pfu DNA polymerase, dNTPs, and the PCR optimization kit were purchased from Stratagene. The DNA primers and oligonucleotides were synthesized by Integrated DNA Technologies and were used without further purification.

L-Aspartic β-semialdehyde (L-ASA) was prepared by the ozonolysis of L-allylglycine according to the method of Black and Wright (1955). Synthesis of 2-amino-5-hydroxy-L-valeric acid was performed by using the procedure of Ramalingam (1988). 1,2-Amino-1,4-butanediol and 1,2-amino-1,5-pentanediol were prepared by methylation of L-aspartic and L-glutamic acids, then reduction with $LiAlBH_4$ by following the procedure of Dickman et al. (1990). L-Threonine α-methyl ester and L-homoserine esters have been prepared by direct esterification with the alcohol present in excess. *N*-Acetyl-L-threonine was synthesized by the method of Overby and Ingersoll (1951). 2-Amino-3-(phosphonomethyl)thiopropionic acid was a generous gift from the late Dr. Fred Wedler.

Enzyme Assays and Data Analysis. Homoserine kinase was purified from a strain of *E. coli* transformed with a thrB-containing plasmid, and the activity was assayed by coupling the production of ADP with pyruvate kinase and lactate dehydrogenase as previously described (Huo & Viola, 1996).

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: ASA, aspartate β-semialdehyde; DEP, diethyl pyrocarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HK, homoserine kinase; HPG, (*p*-hydroxyphenyl)glyoxal; PK/LDH, pyruvate kinase/lactate dehydrogenase.

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Table 1: Substrate Specificity of Homoserine Kinase

substrate	k_{cat}	% k_{cat}	K_m (mM)	k_{cat}/K_m
L-homoserine	18.3 ± 0.1	100	0.14 ± 0.04	184 ± 17
D-homoserine	8.3 ± 1.1	32	31.8 ± 7.2	0.26 ± 0.03
L-aspartate β-semialdehyde	2.1 ± 0.1	8.2	0.28 ± 0.02	7.5 ± 0.3
L-2-amino-1,4-butanediol	2.0 ± 0.5	7.9	11.6 ± 6.5	0.17 ± 0.06
L-2-amino-5-hydroxyvalerate	2.5 ± 0.4	9.9	1.1 ± 0.5	2.3 ± 0.3
L-homoserine methyl ester	14.7 ± 2.6	80	4.9 ± 2.0	3.0 ± 0.6
L-homoserine ethyl ester	13.6 ± 0.8	74	1.9 ± 0.5	7.2 ± 1.7
L-homoserine isopropyl ester	13.6 ± 1.4	74	1.2 ± 0.5	11.3 ± 1.1
L-homoserine *n*-propyl ester	14.0 ± 0.4	76	3.5 ± 0.4	4.0 ± 1.2
L-homoserine isobutyl ester	16.4 ± 0.8	84	6.9 ± 1.1	2.4 ± 0.3
L-homoserine *n*-butyl ester	29.1 ± 1.2	160	5.8 ± 0.8	5.0 ± 0.5

The inherent ATPase activity of the enzyme was measured by omitting homoserine from the assay mixture. All data were fitted to BASIC versions (Enzyme Kinetics Package, SciTech International) of the computer programs of Cleland (1979).

Inhibition and Fluorescence Studies. Analogs of L-homoserine and L-homoserine phosphate were examined as inhibitors in the homoserine kinase reaction by using the PK/LDH coupled assay. The inhibition constants (K_i) were calculated from a least-squares fit (Enzyme Kinetics Package) to Dixon plots (Dixon, 1953) at a fixed concentration of MgATP and varying concentrations of the homoserine analogs. Inhibition pH profile studies were conducted by examining the substrate inhibition by homoserine at different pH values in neutral acid buffer systems.

The fluorescence emission spectra of homoserine kinase, and the series of mutants that were produced, were examined on an Aminco SPF-500 spectrofluorometer. The enzyme samples, in the absence and presence of homoserine, were excited at 295 nm, and the emission spectrum of each was scanned from 310 to 400 nm.

^{31}P NMR Studies of Substrate Specificity. ^{31}P NMR spectra were obtained on a Gemini-300 NMR spectrometer operating at 121 MHz for ^{31}P resonance and equipped with a field-frequency locked on the deuterium resonance. All of the experiments were performed at room temperature with 2−12 h data acquisitions. Each reaction mixture (0.70 mL) contained 100 mM Hepes/Tris, pH 8.0, 100 mM KCl, 3 mM MgATP, 10 mM of either L-homoserine or analogs, 0.1−0.5 unit of HK, and 20% D_2O. All spectra have been referenced to the standard spectrum of 85% phosphoric acid.

Inactivation of Homoserine Kinase. Enzyme inactivation by (*p*-hydroxyphenyl)glyoxal was conducted in 0.1 M sodium bicarbonate buffer, pH 9.0. Activity of the modified enzyme was assayed by removing aliquots at set time intervals. For the stoichiometry determination, 0.55 mg of protein was added to sodium bicarbonate buffer, pH 9.0, and modified in the presence of 40 mM HPG at 30 °C. Aliquots (0.5 mL) were removed at different time intervals and passed down prepacked Sephadex G-25 mini-columns. The protein fraction was collected, activity was assayed, and the absorbance at 340 nm was measured. The stoichiometry of modification was calculated by using an absorption coefficient of 1.83×10^4 M^{-1} cm^{-1} at this wavelength for the product formed from the reaction of 2 mol of reagent per mole of reactive arginine (Yamasaki et al., 1980).

Protein Sequence and Alignment. The amino acid sequences of the homoserine kinases from different organisms shown in this paper were retrieved from either Genbank or Swiss-protein database via the electronic mail server at the National Center for Biological Information of the National Library of Medicine. Multiple sequence alignments were performed by using the program Clustal V (Higgins et al., 1992).

Site-Directed Mutagenesis. Site-directed mutagenesis of the thrB gene for the mutants H202L and R234L was carried out by using Kunkel's method (1987). The single-stranded DNA template has been prepared by cloning the thrB gene into the m13mp18 vector at *Hin*dIII and *Eco*RI sites. The R234C and H139L mutants were generated by PCR on an MJ Research thermal cycler by using the megaprimer method of Barik (1993). To ensure the fidelity of DNA extension, the Taq DNA polymerase was replaced with pfu polymerase. The two PCR primers were designed to amplify a DNA insert between the *Hin*dIII and *Eco*RI sites in the pTZ19 plasmid. The annealing conditions for the primers and the mutagenic oligonucleotides were optimized with the Opti-Prime PCR optimization kit (Stratagene), and megaprimers were generated by using a three-step amplification protocol preprogrammed into the thermocycler. The mutant genes that were generated by each of these methods were then cloned into the pTZ19u vector at the *Hin*dIII and *Eco*RI sites for expression. Oligonucleotides were designed to generate specific site-directed mutations, while at the same time either creating or eliminating a cleavage site for a restriction endonuclease. All of the mutations have been verified by restriction enzyme digestion where possible, and by DNA sequencing of the mutagenic region by using the dideoxynucleotide method (Sanger et al., 1977). Expression and purification of the mutant enzymes have been performed by the method of Huo and Viola (1996). The purity of the proteins was examined by SDS−PAGE gels (Laemmli, 1970) stained with Coomassie Blue, and the protein concentrations have been determined by the method of Bradford (1976).

RESULTS

Specificity of Homoserine Kinase. A wide range of structural analogs of L-homoserine have been examined as possible alternative substrates for the reaction catalyzed by homoserine kinase. Kinetic and ^{31}P NMR studies have shown that the enzyme is able to phosphorylate the hydroxy group on γ-carbon of the L-homoserine analogs when the functional group at the α-position is a carboxyl, an ester, or even a hydroxymethyl group. The enzyme can also phosphorylate D-homoserine at a reasonable rate, although the affinity for this substrate is over 200-fold weaker than that of the L-isomer. The kinetic parameters have been determined for each of these alternative substrates, and the values are compared to those of the physiological substrate (Table 1). Esterification of the substrate leads to a substantial

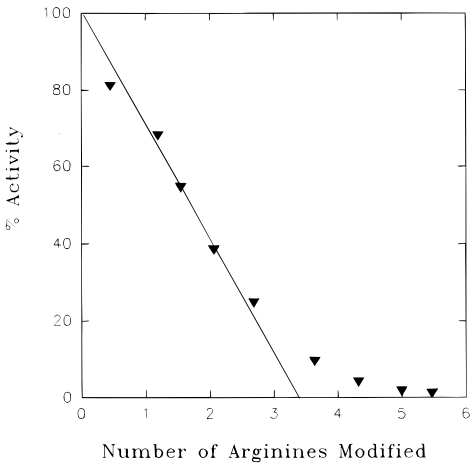

FIGURE 1: Titration of argininyl residues modified by HPG. The modification was made in 0.1 M $NaHCO_3$ buffer, pH 9.0. To a total volume of 2.5 mL was added 0.55 mg of protein with 40 mM HPG. The mixture was incubated at 30 °C; 0.5 mL aliquots were removed at different time intervals. The excess of HPG was removed by size-exclusion chromatography; the enzyme activity and absorbance at 340 nm were determined.

increase in the K_m values of the compounds for the enzyme; however, there is little or no decrease in the phosphorylation rate regardless of the size of the ester. Among the alternative substrates, 1,2-amino-5-hydroxyvalerate and L-homoserine α-methyl ester show substrate inhibition, as does the physiological substrate. Unlike aspartokinase I (Angeles et al., 1992) or aspartokinase III (Keng & Viola, 1996), enzymes which catalyze the first step of the aspartate pathway, HK cannot reverse regiospecificity to phosphorylate a hydroxyl group located on the α-carbon of these substrate analogs, and the enzyme is also unable to phosphorylate either thiol or amino groups. A number of amino acids and amino diols with a five-carbon skeleton or shorter have been found to be reasonably good inhibitors of the enzyme. Among the better inhibitors of HK are L-norvaline, L-alanol, L-glutamic acid, L-threonine, and L-cysteine, all with K_i values in the range of 0.2−0.5 mM. Compounds that are missing the α-carboxyl group (3-amino-1-butanol), missing the α-amino group (4-hydroxybutyrate), or have a modified α-amino group (*N*-acetylthreonine) are either weak inhibitors or noninhibitors of the enzyme.

For homoserine phosphate analogs, *O*-phosphoserine is a moderate inhibitor (K_i = 2.7 mM), while the phosphonate analog of this product, 2-amino-5-phosphonovalerate (with $-OPO_3^{2-}$ replaced by $-CH_2PO_3^{2-}$), shows weaker inhibition (K_i = 10.4 mM). However, 2-amino-3-(phosphonoethyl)thiopropionate ($-CH_2OPO_3^{2-}$ replaced by $-S-CH_2PO_3^{2-}$) is a fairly good inhibitor of HK (K_i = 0.3 mM).

Inactivation of Homoserine Kinase. (*p*-Hydroxyphenyl)glyoxal (HPG) is an arginine-specific reagent (Yamasaki et al., 1980), with 2 mol of reagent required for the modification of each mole of argininyl residue (Lundblad, 1995). The enzyme was incubated with different levels of HPG, and a time-dependent, but nonlinear inactivation of the enzyme was observed. Higher pH led to increased inactivation initially, but a plot of log (V_t/V_0) vs time leveled off after a short period of time. A curve of enzyme activity vs stoichiometry of modification is plotted in Figure 1. Extrapolation of this curve extends to the modification of between three and four rapidly reacting arginine residues per subunit with complete loss of enzyme activity. Each of the substrates offers moderate protection against inactivation, with the highest protection afforded by the ternary complex of MgATP and threonine with HK. When the inactivation is carried out in the presence of 10 mM L-homoserine, approximately one arginine residue per subunit is protected against modification, and the enzyme retains about 60% activity. The ternary MgATP/threonine complex also protects one arginine against modification.

Selection of Mutagenesis Targets. The choice of the appropriate amino acid targets for site-directed mutagenesis was based on the results of pH and chemical modification studies of this enzyme, sequence comparisons among related enzymes, and the hypothesis that proteins with the same function have similar tertiary structures (Rossmann et al., 1975). The amino acid sequences of HK from various species were aligned by using the Clustal V program. There are several regions of homology among the HK enzymes, including several groups of conserved functional amino acids, and a putative ATP-binding domain consisting of a PxGxGLGSS sequence. Inactivation studies with HPG have suggested the importance of an argininyl residue in the catalytic activity of HK. There is 1 argininyl residue conserved in 9 of the 10 sequences, and arginine-234 is also located in a highly conserved region (Figure 2). Previous inactivation studies (Huo & Viola, 1996) have indicated possible roles for lysyl and histidinyl residues in HK. None of the 11 lysines in the *E. coli* enzyme are found in the same position in more than 2 of the other 9 HK sequences. The 6 histidinyl residues in *E. coli* HK are also not fully conserved; however, histidine-139 is conserved in 9 of the 10 sequences (with a tyrosine replacing the histidine at this position in the enzyme from *Pseudomonas aeruginosa*). Histidine-202 is conserved in 3 sequences, with the position in 5 of the other 8 sequences occupied by an asparaginyl residue, and histidine-205 is conserved in 3 of the sequences with an arginine at this position in 5 other sequences (Figure 2). These three histidines and arginine-234 were selected as the most promising positions to be examined by site-directed mutagenesis.

Characterization of the Homoserine Kinase Mutants. Arginine-234 was first replaced with the neutral amino acid leucine. The K_m of R234L for L-homoserine increases by nearly 300-fold, and the k_{cat} decreases by 90-fold compared to the wild-type enzyme (Table 2). There is, however, less than a 2-fold change in the K_m for ATP, and the inherent ATPase activity of homoserine kinase (<0.1% of the kinase activity) actually increases by 3-fold in the R234L mutant. In contrast to the lack of selectivity in the wild-type enzyme, this mutant has k_{cat} values for homoserine esters that are only 10% that of homoserine, but has a higher affinity for the esters than for L-homoserine itself. The K_m values for the ethyl and the propyl esters of homoserine are in the range of 1.0−1.5 mM, 40 times lower than that of L-homoserine. This leads to k_{cat}/K_m values for homoserine esters with the R234L mutant of HK that are comparable or even higher than that of the physiological substrate (Table 3). Inhibition studies also confirm that this mutant enzyme now has a preference for alkyl, hydroxymethyl, or ester functional groups over an anionic carboxyl group at the α-position of the substrate analogs. L-Cysteine, a strong inhibitor of the wild-type enzyme (K_i = 0.46 mM), is 50-fold less effective as an inhibitor of R234L, and L-threonine (K_i = 0.30 mM)

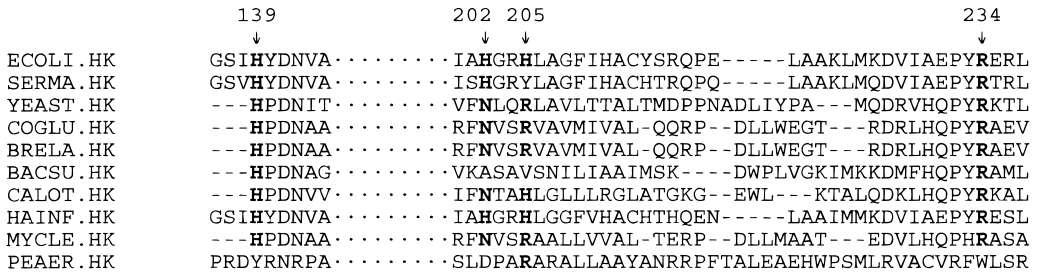

FIGURE 2: Sequence alignment of the core region of the homoserine kinase from different sources. The position numbers on the top of each block are those of the *E. coli* HK. The species abbreviations used are as follows: ECOLI, *Escherichia coli*; YEAST, yeast (*Saccharomyces cerevisae*); SERMA, *Serratia marcescens*; COGLU, *Coryneacteriuum glutamicum*; BRELA, *Brevibacterium lactofermentum*; BACSU, *Bacillus subtilis*; CALOT, *Calothrix* PCC 760; PEAER, *P. aeruginosa*.

Table 2: Kinetic Parameters of Homoserine Kinase Mutants

enzyme	k_{cat} (s^{-1}) kinase	k_{cat} (s^{-1}) ATPase	kinase/ATPase ratio	K_{mATP} (mM)	K_{mHSE} (mM)	K_{iHSE} (mM)
wild type	18.3 ± 0.1	0.016 ± 0.002	1140	0.13 ± 0.01	0.14 ± 0.04	6.9 ± 1.8[a]
R234L	0.20 ± 0.02	0.049 ± 0.008	4.1	0.21 ± 0.03	40.1 ± 8.8	nd[b]
R234C	0.022[c]	0.28 ± 0.08	0.08	0.88 ± 0.29	—[d]	8.5 ± 3.9[e]
R234H	0.073 ± 0.001	0.37 ± 0.01	0.19	0.13 ± 0.02	0.13 ± 0.08	6.2 ± 3.6[a]
H139L	0.52[c]	2.51 ± 0.014	0.21	0.49 ± 0.09	—[d]	2.5 ± 1.1[e]
H202L	9.06 ± 0.06	0.071 ± 0.011	128	0.11 ± 0.02	0.11 ± 0.02	58.2 ± 13.0[a]
H205Q	0.005[c]	0.036±0.006	0.14	0.15 ± 0.03	—[d]	3.7 ± 0.4[e]

[a] Determined from substrate inhibition of the kinase activity. [b] Not detected. [c] Estimated as described under Experimental Procedures from the partition ratio of kinase to ATPase activity determined by phosphorus-31 NMR spectroscopy. [d] Cannot be directly measured due to the mixed ATPase and HK activities. [e] Determined from inhibition of the ATPase activity.

Table 3: Alternative Substrates for Homoserine Kinase Mutants

substrate[a]	k_{cat} (s^{-1}) H202L	k_{cat} (s^{-1}) R234L	k_{cat}/K_m H202L	k_{cat}/K_m R234L
L-homoserine	9.1 ± 0.1	0.20 ± 0.02	82.7 ± 0.9	0.0050 ± 0.0005
L-homoserine methyl ester	5.4 ± 0.6	0.018 ± 0.002	3.0 ± 0.3	0.0025 ± 0.0003
L-homoserine ethyl ester	4.1 ± 0.2	0.021 ± 0.003	3.0 ± 0.2	0.0190 ± 0.0003
L-homoserine propyl ester	2.7 ± 0.2	0.011 ± 0.001	1.4 ± 0.1	0.0075 ± 0.0007
L-homoserine butyl ester	2.5 ± 0.4	0.007 ± 0.002	2.3 ± 0.4	0.0007 ± 0.0001

no longer inhibits this mutant enzyme. The R234L mutant is still sensitive to modification with the arginine reagent HPG; however, unlike the wild-type enzyme, the addition of 10 mM L-homoserine to this mutant has no protective effect on the number of argininyl residues titrated by HPG.

Arginine-234 was also replaced with cysteine and histidine. The R234C mutant has no observable homoserine kinase activity by using the coupled enzyme assay; however, it now has an ATPase activity that is nearly 20 times that of the wild-type enzyme at pH 8.0. It is not possible to directly measure a kinase activity for this mutant in the presence of the enhanced ATPase activity. However, when the time course for the enzyme-catalyzed reaction is followed by phosphorus-31 NMR, a peak is observed that corresponds to the production of homoserine phosphate. Integration of this peak relative to the inorganic phosphate produced by the enzyme-catalyzed hydrolysis of ATP allows quantitation of the kinase activity of R234C at about 10% that of the enhanced ATPase activity. When corrected for the inhibition of ATPase activity in the presence of homoserine, a k_{cat} value is calculated for the kinase activity of this mutated enzyme which is about 0.1% that of the wild-type enzyme (Table 2). The R234H mutant also has diminished kinase activity (0.4% of wild type) and an enhanced ATPase activity. The K_m values for both substrates are unchanged in the presence of a histidine at position 234, while a cysteine at this position results in a 7-fold increase in K_{ATP}.

Unexpectedly, when the number of cysteines in the R234C mutant are quantitated by DTNB titration, three fewer cysteines are detected than in the wild-type enzyme, instead of the one additional cysteine that was anticipated. This R234C mutant enzyme is also very sensitive to heat treatment and begins to precipitate at 55 °C. The intensity of the relative fluorescence at 340 nm is only about 30% that of the wild-type enzyme under the same conditions (data not shown), and the addition of up to 20 mM L-homoserine has no effect on the fluorescence spectrum.

Mutation of histidine-139 to leucine leads to an enzyme with dramatically enhanced ATPase activity, about 150-fold greater than that of the wild-type enzyme, and with diminished kinase activity (Table 2). When calculated from the ratio of kinase to ATPase products by using phosphorus-31 NMR spectroscopy as described above, the kinase activity of H139L is less than 3% that of the wild-type enzyme. The H139L mutant enzyme has a similarly diminished fluorescence spectrum to that of the R234C mutant; however, the addition of homoserine results in a 10% decrease in the fluorescence intensity for this mutant.

Replacing histidine-202 with leucine leads to a decrease in k_{cat} by only 50%. The K_m values for L-homoserine and for ATP remain unchanged, but the K_i for the substrate inhibition by L-homoserine increases by about 8-fold. The relative fluorescence of the H202L mutant is essentially the same as that of the wild-type enzyme, indicating that there

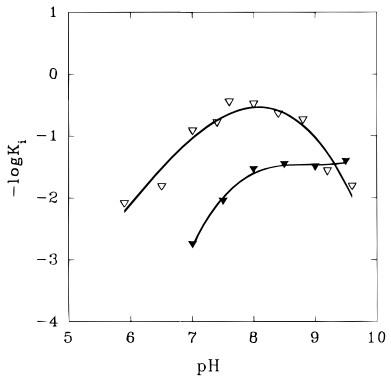

FIGURE 3: pH profile of the substrate inhibition of homoserine kinase. (▽) Wild-type enzyme; (▼) H202L mutant.

is no major change in the protein structure when this mutation is introduced. The substrate specificity is essentially the same as that of the wild-type enzyme (Table 3); however, unlike the wild-type enzyme the L-homoserine ethyl, isopropyl, and *n*-propyl esters now show substrate inhibition with the H202L mutant enzyme. The V_{max} and V/K pH profiles of the H202L mutant are essentially unchanged from the wild-type enzyme (data not shown), but the basic side of the substrate inhibition pH profile is flat with no decrease in the K_i for L-homoserine at higher pH (Figure 3).

Replacement of histidine-205 with glutamine leads to an enzyme with an unaltered K_m for ATP and an ATPase activity that is within a factor of 2 of the wild-type enzyme. However, the kinase activity of H205Q, as determined from phosphorus-31 NMR, is less than 0.03% that of wild-type enzyme (Table 2). The H205Q enzyme has a maximal fluorescence intensity (at 340 nm) that is 60% that of the wild-type enzyme. Addition of homoserine leads to the quenching of the fluorescence emission spectrum of HK. Titration of this quenching allows a determination of the dissociation constants for homoserine, and the changes in these values for the mutants of HK are comparable to the changes that have been observed for the K_m values.

DISCUSSION

Specificity of Homoserine Kinase. An examination of a wide range of structural analogs of homoserine has shown that an intact amino group is absolutely essential for effective binding at the active site of HK. The enzyme does, however, allow a considerably wider latitude for the carboxyl and hydroxyl determinants of the amino acid substrate. The enzyme is able to phosphorylate the hydroxyl group at either the β- or the γ-carbons of L-homoserine analogs, but neither L-threonine nor L-serine are substrates of the enzyme. For four-carbon L-homoserine analogs, the carboxyl group at the α-position can be a carboxyl, or could be substituted with an ester or even a hydroxymethyl group. The enzyme can also accommodate large, hydrophobic groups at the carboxyl end of the substrate. L-Homoserine esters with ester groups up to four carbons long are processed without a substantial sacrifice in catalytic efficiency, although the selectivity (k_{cat}/K_m) for homoserine is 20−80-fold higher than for homoserine esters. These results suggest the presence of a hydrophobic pocket adjacent to the binding site of the carboxyl group of the substrate on the active site, with hydrophobic interactions between the alkyl groups and the surrounding pocket at least partially compensating for the loss of an ionic interaction to aid in the productive orientation of these alternative substrates.

Identification of Functional Amino Acid Residues. Arginines are frequently found to be involved in the binding of anionic groups in proteins (Riordan, 1979; Haining & McFadden, 1990). A sequence comparison of homoserine kinases from different organisms shows that among the 20 arginines in the *E. coli* enzyme only arginine-234 is highly conserved, and it is also located in a highly conserved region among the sequences of all homoserine kinases. Replacement of this argininyl residue with a neutral leucine results in dramatic increase in the K_m value for L-homoserine and a substantial decrease in k_{cat}. In addition to these altered kinetic parameters, this R234L mutant also has altered substrate specificity, with a preference for the binding of substrate analogs with a neutral or polar group at the α-position over the anionic group of the physiological substrate. This altered binding specificity, and the fact that L-homoserine does not protect against the modification of the mutant by HPG, strongly suggests that arginine-234 is directly involved in an ionic interaction with the α-carboxyl group of the substrate to help properly position the substrate for productive phosphorylation.

Replacement of this argininyl residue with a cysteinyl group leads to a further 10-fold loss of enzymatic activity compared to the already compromised R234L mutant. An increased sensitivity to heat treatment and a diminished fluorescence spectrum demonstrate that the protein is structurally altered as compared to the wild-type enzyme. A decrease in the number of titratable cysteinyl residues supports the idea that the introduction of an additional cysteinyl residue leads to the formation of either intra- or intersubunit disulfide bonds which have contributed to a conformational change that has altered the active site structure.

The functional role of several histidine residues has also been examined. Replacing histidine-202 with a leucine group has little effect on the fluorescence spectrum of HK, suggesting that this replacement does not result in significant structural alterations in the enzyme. The substrate inhibition studies with L-homoserine, and the earlier kinetic studies (Shames & Wedler, 1984), have suggested that there are two binding sites for homoserine, one at the active site and the other at a regulatory site. The observation that the basic pK of the substrate inhibition profile is eliminated in the H202L mutant (Figure 3) while the k_{cat} and K_m values are not significantly altered shows that histidine-202 is involved in binding at the regulatory site of the enzyme, and also provides evidence that the regulatory site and the active site are separate spatially resolved entities. Increased affinity toward L-homoserine esters at the regulatory site by the mutant enzyme suggests that histidine-202 is most probably involved in the binding of the carboxyl group of L-homoserine at that site.

Replacement of histidine-205 has no effect on the binding of ATP or on the inherent ATPase activity of HK. The kinase activity of the enzyme is, however, decreased by over 3000-fold by this substitution. The removal of histidine-139 also leads to a dramatic decrease in the k_{cat} for phosphoryl transfer to homoserine, but results in a 150-fold enhancement in the rate of phosphoryl transfer to water. This mutation has led to a compromise in the active site structure that has allowed solvent facile access to compete with

homoserine as the phosphoryl acceptor. For each of these mutations, the observed decrease in the fluorescence emission spectra indicates some structural rearrangements. However, these structural alterations are only manifested in changes in the homoserine binding region of the active site of HK. There is no evidence for the synergism in substrate binding that has been observed in other kinases such as hexokinase (Viola et al., 1982), fructokinase (Raushel & Cleland, 1977), and creatine kinase (Maggio et al., 1977). The decoupling of the kinase and the ATPase activities of this enzyme through specific site-directed mutations suggests the presence of independent domains for the binding of each substrate in homoserine kinase.

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