Pyridoxal 5'-Phosphate Dependent Histidine Decarboxylase: Overproduction, Purification, Biosynthesis of Soluble Site-Directed Mutant Proteins, and Replacement of Conserved Residues†

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ABSTRACT: The *hdc* gene coding for the pyridoxal 5'-phosphate dependent histidine decarboxylase from *Morganella morganii* has been expressed in *Escherichia coli* under control of the *lac* promoter. The enzyme accumulates to 7–8% of total cell protein and is purified to homogeneity by passage through three columns. Fourteen site-directed mutant enzymes were constructed to explore the roles of residues of interest, especially those in the sequence Ser^{229}-X^{230}-His^{231}-N^{ϵ}-(phosphopyridoxylidene)Lys^{232}, since identical sequences also appear in several other decarboxylases. Most of the overproduced mutant proteins were aggregated into inclusion bodies, but when the late log phase cultures were cooled from 37 to 25 °C before induction, the mutant proteins were obtained as soluble products. Ala or Cys in place of Ser-229 yielded mutant enzymes about 7% as active as wild-type, indicating that this serine residue is not essential for catalysis but contributes to activity through conformational or other effects. Of the replacements made for His-231 (Asn, Gln, Phe, and Arg), only Gln and Asn gave partially active enzymes (about 12% and 0.2% of wild-type, respectively). The side-chain amide of Gln may act by mimicking the positionally equivalent τ-nitrogen on the imidazole ring of histidine to provide an interaction (e.g., a hydrogen bond) required for efficient catalysis. The Lys-232 residue that interacts with pyridoxal 5'-phosphate appears central to catalytic efficiency since replacing it with Ala yields a mutant protein that is virtually inactive but retains the ability to bind both pyridoxal 5'-phosphate and histidine efficiently. None of four other residues (Met-233, Cys-240, Cys-329, Ser-322) chosen for replacement because of their possible oxidative lability or role in catalysis proved essential for activity, although all replacements reduced the activity of the enzyme significantly.

The pyridoxal 5'-phosphate (PLP)[1] and pyruvoyl-dependent histidine decarboxylases (HisDCases)[2] represent a rare example of independent evolution leading to very different proteins that catalyze an identical reaction. So far, all HisDCases that have been isolated from Gram-negative bacteria (Guirard & Snell, 1987; Tanase et al., 1985) or mammals (Feldberg et al., 1988; Martin & Bishop, 1986; Taguchi et al., 1984) use PLP as a coenzyme and have identical subunits of M_r 43 000–55 000 while those from Gram-positive bacteria have a pyruvoyl prosthetic group and have dissimilar large and small subunits [see Recsei and Snell (1984) and references cited therein]. Representative examples of the two classes of HisDCases are the PLP-dependent enzyme from *Morganella morganii* AM15 and the pyruvoyl enzyme from *Lactobacillus* 30a. Each has been extensively studied [see Recsei and Snell (1984) and references cited therein; van Poelje & Snell, 1988; Tanase et al., 1985; Hayashi et al., 1986]; they are equally efficient catalysts, but their pH optima, K_m values, and sensitivity to inhibitors differ. A comparison of the architecture and the mechanism of action of the two enzymes would illustrate how different structures can perform the same function.

The amino acid sequence of the pyruvoyl enzyme from *Lactobacillus* 30a, determined by Huynh et al. (1984a,b) and corrected in several details by sequencing of the gene (Vanderslice et al., 1986), permitted fitting of the sequence to the X-ray crystallographic structure of this enzyme at 3-Å resolution (Parks et al., 1985). The unrelated amino acid sequence of the PLP-dependent HisDCase from *M. morganii* was deduced from the nucleotide sequence of the *hdc* gene (Vaaler et al., 1986). The purpose of this work is 3-fold: (1) to develop an expression system for the overproduction of the PLP-dependent HisDCase in *Escherichia coli*; (2) to purify the overproduced enzyme; and (3) to use site-directed mutagenesis to study the role of selected amino acid residues. These include particularly residues near the Lys-232 which is in Schiff base linkage with PLP; a Ser-X-His-(P-Pxd)Lys sequence is present in the active-site peptides from PLP-dependent glutamate, arginine, and lysine decarboxylases from *E. coli* (Boeker & Snell, 1972), and in the tryptophan decarboxylase from *Catharanthus roseus* (De Luca et al., 1989). Further objectives include (a) study of the role of Ser-322 in the irreversible "suicide" inactivation of this enzyme by α-(fluoromethyl)-histidine (Hayashi et al., 1986) and (b) construction of a mutant enzyme that will form crystals suitable for X-ray crystallography. Wild-type HisDCase from *Morganella* has been crystallized (M. L. Hackert, personal communication), but the crystals are fragile and crack upon exposure to air and/or manipulation.

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[1] Abbreviations: PLP, pyridoxal 5'-phosphate; HisDCase, histidine decarboxylase; P-Pxd, 5'-phosphopyridoxylidene group; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; kbp, kilobase pairs; Ap^r, resistance to ampicillin; IPTG, isopropyl β-D-thiogalactopyranoside; His, histidine, PL, pyridoxal; Xgal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

[2] L-Histidine carboxy-lyase (EC 4.1.1.22). The enzyme commission makes no distinction between the PLP and pyruvoyl-dependent enzymes.

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Table I: Bacterial Strains, Phage, and Plasmids

bacterial strains	genotype	source (ref)
E. coli K-12		
GM1	*ara* Δ(*lacpro*)*thi*/F′(*lacpro*)*lacI*^{q}L8	R. J. Meyer (Miller et al., 1977)
BW313	HfrKL16PO/45[*lysA*(61–62)] *dut1 ung1 thi1 relA1*	T. O. Baldwin (Kunkel, 1985; Kunkel et al., 1987)
JM103Y	Δ(*lacpro*)*thi strA supE endA sbcB15 hsdR4*/F′*traD36 proAB lacI*^{q}ZΔM15	T. O. Baldwin (Messing et al., 1981)
RB791 (=W3110)	*lacI*^{q}L8	R. J. Meyer (Amann et al., 1983)
JM107	Δ(*lacproAB*)*thi gyrA96 endA1 hsdR17 relA1 supE44* λ$^-$/F′;*traD36 proAB lacI*^{q}ZΔM15	D. Appling (Yanisch-Perron et al., 1985)
TG1	JM101 *ecoK*$^-$ Δ(*lacpro*) *supE thi hsdD5*/F′*traD36 proAB lacI*^{q}ZΔM15	D. Appling (Carter et al., 1985)
Morganella morganii AM-15 (ATCC 35200)		M. Miyaji, Chiba University, Japan

phage and plasmids	relevant properties	source or construction
M13mp18		Bethesda Research Labs (Yanisch-Perron et al., 1985)
mGV24	*hdc* gene inserted reversed with ref to *lac* promoter	insertion of 1.3 kbp *Nae*I fragment from pGV1 (Vaaler et al., 1986) into M13mp18 cut with *Sma*I
pGV37	Ap^r, expression plasmid for wild-type HisDCase	insertion of 1.3 kbp *Eco*RI–*Hin*dIII fragment from mGV24 into pUC9 cut with *Eco*RI and *Hin*dIII
pGV36	Ap^r	as for pGV37, insertion into pUC8 for reverse orientation of gene
pGV39-pGV52	Ap^r, expression plasmids for mutant HisDCases	site-specific mutagenesis of single-stranded mGV24 with oligonucleotides in Table II; after screening, construction as for pGV37

In pursuing these goals, we found many of our overproduced mutant proteins present in insoluble inclusion bodies. A common strategy for dealing with this densely aggregated protein is to pellet the granules, solubilize the protein under denaturing conditions, and search for conditions under which the protein will refold into its native state. If this strategy works, inclusion body formation is an efficient purification step. For many enzymes, including this decarboxylase, the renaturation process is neither efficient nor complete and would obscure the effect of the mutation on structure and function. We describe here a low-temperature induction that allows overproduced mutant proteins to be synthesized and obtained as soluble products.

Experimental Procedures

Materials. The bacterial strains, phage, and plasmids used or constructed for this work are described in Table I. Strains carrying the *hdc* wild-type or mutant genes were streaked onto plates containing minimal A medium with glucose (Miller, 1972) (for selection of the F′*lacpro*) and ampicillin and then grown in TYE medium containing 1.0% tryptone, 0.5% yeast extract, 0.5% sodium chloride, and 200 μg/mL ampicillin. *Morganella* was grown as described by Tanase et al. (1985). *E. coli* JM103Y was grown overnight in minimal medium prior to dilution into YT medium (Miller, 1972). *E. coli* BW313 was grown in YT medium (Miller, 1972). The oligonucleotides used for site-directed mutagenesis and DNA sequencing were synthesized on an Applied Biosystems Model 380A or 381A DNA synthesizer. Table II lists the codon change, the position of the mutagenic oligonucleotide, and the resultant amino acid change for each mutant constructed for this work. Oligonucleotides used for mutagenesis were designed to contain the base(s) to be changed close to the middle and the 3′ base(s) to be C or G to enhance binding where DNA synthesis is to begin. The oligonucleotides were partially purified by passage through Sephadex G50 as described in the Applied Biosystems User Bulletin (1984). Sequencing reagents, restriction endonucleases, polynucleotide kinase, T4 DNA ligase, and Polymin P were purchased from New England Biolabs and Bethesda Research Laboratories. Other chemicals and their sources were as follows: Klenow fragment, Boehringer Mannheim; T4 DNA polymerase and T4 gene 32 protein, Bio-Rad; Sequenase, U.S. Biochemical Corp.; deoxynucleoside triphosphates (HPLC grade, 100 mM solutions) and aminohexyl-Sepharose 4B, Pharmacia; [α-^{35}S]dATP (1000–1500 Ci/mmol) and L-[1-^{14}C]histidine (30–60 mCi/mmol), New England Nuclear; DEAE-cellulose, Whatman; Sephacryl S-300, Sigma. All other reagents were of the highest quality available.

Table II: Oligonucleotides Used for Directed Mutagenesis

mutation[a]	codon change[b]	nucleotide position of mutagenic primer[c]
S229A	TCC → GCC	681 → 702
S229C	TCC → TGC	681 → 702
H231R	CAC → CGC	688 → 707
H231F	CAC → TTC	688 → 707
H231Q	CAC → CAG	688 → 707
H231N	CAC → AAC	688 → 707
K232A	AAA → GCA	689 → 707
M233I	ATG → ATC	692 → 712
C240A	TGC → GCC	710 → 730
C240S	TGC → TCC	710 → 730
S322A	TCC → GCC	957 → 976
S322T	TCC → ACC	957 → 976
C329A	TGC → GCC	981 → 1001
C329S	TGC → TCC	981 → 1001

[a] Mutant proteins are specified by indicating in order the wild-type residue, its position in the sequence, and the replacing residue. In S229A, for example, Ser-229 has been changed to Ala. [b] The nucleotide change is underlined. [c] Nucleotide 1 is the A of the formylmethionine codon. See Vaaler et al. (1986) for the nucleotide sequence of the *hdc* gene.

Expression Vector Construction. All DNA manipulations were carried out by using standard procedures (Maniatis et al., 1982). The coding region for *Morganella* HisDCase is contained on a 1.3 kbp *Nae*I fragment on plasmid pGV1 (Vaaler et al., 1986). An *Nae*I digest of pGV1 was ligated to a *Sma*I digest of M13mp18. Following transformation, the desired recombinant phage, mGV24, was identified by restriction endonuclease mapping. This construct contains the *hdc* gene on an *Eco*RI–*Hin*dIII fragment which when cloned into pUC9 and pUC8 positions the gene in the correct orientation with reference to the *lac* promoter (constructing pGV37) and in the reverse orientation (constructing pGV36). These plasmids were transformed into a number of host strains and plated without IPTG and Xgal in case the expression of HisDCase was deleterious. Ap^r transformants containing the

1.3 kbp *Eco*RI–*Hin*dIII fragment were identified by restriction endonuclease mapping.

Induction of HisDCase. Elucidation of optimal induction conditions was achieved by assaying cells permeabilized with 4% toluene and 0.004% deoxycholate for HisDCase activity. Late logarithmic phase cultures with an A_{550} of 1.5–2.0 were induced with 0.5 mM IPTG for 90 min at 37 °C. Pyridoxal (5 μg/L), histidine (0.05%), and additional ampicillin (100 μg/mL) also were added. Overproduction of mutant enzymes was achieved by induction at 25 °C for 4 h, and the amount of soluble HisDCase was compared to that formed by inducing at 37 °C for 1.5 h.

Purification. Harvested cells were washed in extraction buffer (50 mM KH_2PO_4, 50 mM succinic acid, 2 mM EDTA, 1 mM DTT, and 10 μM PLP, pH 6.0). The wet weight was determined, and the pellet was frozen until use. A pellet (~10.6 g) from three 1-L cultures was resuspended in 45 mL of extraction buffer and passed through the French press twice at 1500 lbs. Lysis was confirmed by microscopic examination of the extract. Aqueous 5% (v/v) Polymin P (adjusted to pH 6.0 with HCl) was added dropwise to a final concentration of 0.2%. The suspension was stirred for 30 min on ice and centrifuged at 100000*g* for 45 min, and the pellet was discarded. The fraction of the supernatant soluble in 35% ammonium sulfate but insoluble in 70% ammonium sulfate was collected and dissolved in ~15 mL of buffer A [50 mM KH_2PO_4, 50 mM succinic acid, 2 mM EDTA, 0.1 mM DTT, 0.01 mM PLP, 5% glycerol, and 0.009% (hydroxypropyl)-cellulose, pH 5.8]. After dialysis against two changes of buffer A (4 L overnight and 2 L for 2 h), the sample was diluted to contain ~15 mg of protein/mL and loaded onto an aminohexyl-Sepharose 4B column (1.7 × 23 cm) equilibrated with buffer A without PLP. The column was washed with 1 L of buffer A with PLP and then with a linear gradient of 0–0.5 M KCl in buffer A (175 mL in each bottle). Active fractions were pooled and concentrated by ultrafiltration. This fraction was dialyzed against 4 L of buffer B [25 mM succinic acid, 0.1 mM EDTA, 0.1 mM DTT, 5 μM PLP, 5% glycerol, and 0.009% (hydroxypropyl)cellulose, pH 5.8] and applied to a DEAE-cellulose column (2.5 × 20 cm) equilibrated with buffer B. This column was washed with ~200 mL of buffer B and then with a linear gradient of 0–0.35 M KCl in buffer B (150 mL in each bottle). Active fractions were pooled and concentrated to ~10 mg/mL for application to the Sephacryl S-300 column (2.0 × 74 cm) equilibrated with buffer C [50 mM succinic acid, 0.1 mM EDTA, 0.1 mM DTT, 5 μM PLP, 5% glycerol, and 0.009% (hydroxypropyl)cellulose, pH 6.0]. Batches containing about 20 mg of protein were loaded and eluted with buffer C. Peak fractions were combined and stored at −20 °C. All purification steps were carried out at 4–6 °C.

HisDCase Assay. HisDCase activity was determined by measuring the rate of $^{14}CO_2$ release from L-[1-^{14}C]histidine as described by Tanase et al. (1985) with a few modifications. (Hydroxypropyl)cellulose was included at 0.17 mg/mL, the volume of the assay mixture was 0.91 mL, and substrate was added last to begin the reaction. After 15 min, the assay was terminated by the addition of 0.5 mL of 1 N HCl.

Protein Determination. Protein concentration was determined by a modified Lowry method (Scopes, 1982) with fraction V BSA as a standard, or for pure HisDCase, by spectrophotometry [$A^{1\%}_{278nm}$ = 14.5 (Tanase et al., 1985)].

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Ten percent acrylamide gels were run, stained with Coomassie Blue R (Fairbanks et al., 1971), and photographed.

Characterization of Recombinant HisDCase. Native and denaturing acrylamide gels were run on the enzyme from *Morganella* and the recombinant enzyme from *E. coli*. Carboxymethylation of the purified recombinant HisDCase was performed as described by Huynh et al. (1984a) and an aliquot hydrolyzed in 6 N HCl at 110 °C for amino acid analysis. For automatic sequencing, a Beckman 890C liquid-phase sequencer equipped with a Sequemat P-6 automatic converter was used. Ouchterlony analysis was performed with antibodies to the *Morganella* enzyme (Guirard & Snell, 1987).

Site-Directed Mutagenesis. For site-directed mutagenesis, the phage mGV24 was transformed into *E. coli* BW313 (*dut*$^-$, *ung*$^-$) (Kunkel, 1985), and uracil-containing single-stranded DNA was purified (Carlson & Messing, 1984). Mutagenic primers were phosphorylated according to Maniatis et al. (1982) and annealed to the uracil-containing template in a 4:1 molar ratio. Heteroduplex formation was performed according to Kunkel et al. (1987) but without ligase, and the reaction mix was transfected into competent *E. coli* JM103Y (*ung*$^+$) cells. Resultant phage were plaque-purified and grown on JM103Y, and single-stranded DNA was purified and screened for the desired mutation by single-lane DNA sequencing.

DNA Sequencing. DNA sequence analysis used the chain-terminating reactions described by Sanger et al. (1977). The complete sequence of each mutant gene was determined in M13 using oligonucleotide primers that annealed at different gene locations. Plasmid DNA was purified in a CsCl gradient and linearized with *Eco*RI before being annealed to the sequencing primers (Wallace et al., 1981).

Expression of Mutant HisDCases. The mutant genes were expressed by cloning them from M13mp18 into pUC9 as described for the wild-type gene. Transformation, screening of colonies, and induction were also as described for wild-type. Cells from a 1.5-L culture were harvested, washed, and lysed in the French press as described, and the crude extract was centrifuged at 100000*g* without the Polymin P treatment. Samples containing about 150 μg of protein of the crude extract, the resuspended pellet, and the supernatant were run on SDS–PAGE. Purification of mutant enzymes was carried out as for wild-type.

Determination of HisDCase on SDS–PAGE. HisDCase bands on photographs were quantified by using a video area densitometer and software under development by L. L. Poulsen and D. M. Ziegler at the Clayton Foundation Biochemical Institute, University of Texas. A standard curve was constructed to relate band intensity to quantity of HisDCase present. In addition, a 10-μg sample of pure HisDCase was run on each experimental gel as a reference for band intensity and size due to differing gel and photographic conditions.

Determination of PLP Content and K_d. Pure wild-type and K232A proteins were dialyzed against 4 L of 0.05 M potassium succinate buffer, pH 6.0, and analyzed for PLP content by reaction with phenylhydrazine (Wada & Snell, 1961). A dissociation constant for histidine was determined spectrophotometrically for the K232A protein by using the absorbance of the wild-type HisDCase at 418 nm (due to its internal azomethine link) as the reference value for 100% Schiff base formation.

Results

Expression Vector Construction. Upon cloning the 1.3 kbp *Nae*I fragment containing the HisDCase gene into M13mp18 (constructing mGV24), we found the fragment present only in the reversed orientation to that needed for expression from the *lac* promoter. This suggested that expression of the gene may be deleterious to the cells. We therefore selected *E. coli*

Table III: Regulation of the Overproduction of HisDCase

strain/plasmid	sp act.[a] [μmol min^{-1} (mL of culture)$^{-1}$] uninduced	induced[b]	inducer
E. coli GM1/pUC9	0.00	0.00	IPTG/His/PL
E. coli GM1/pGV37[c]	0.65	5.41	IPTG/His/PL
E. coli GM1/pGV36[d]	0.64	0.25	IPTG/His/PL
M. morganii AM-15	0.01	0.31	histidine

[a] The assay was done on permeabilized cells. [b] The *E. coli* cultures were induced in late log phase for 1.5 h at 37 °C. The *M. morganii* culture contained histidine in the growth medium (Tanase et al., 1985). [c] *hdc* gene in correct orientation to *lac* promoter. [d] *hdc* gene in reverse orientation to *lac* promoter.

GM1 which has the strong *lac*I^{q}L8 mutation on the F′ episome and maximally produces *lac* repressor as the host strain. We then forced the orientation of the gene by cloning the *Eco*RI–*Hin*dIII fragment into both pUC8 (constructing pGV36 which has the gene reversed to the *lac* promoter) and pUC9 (constructing pGV37 which has the gene in the correct orientation for expression from the *lac* promoter) without using IPTG in the agar plates. This construct, pGV37, contains 116 nucleotides of *Morganella* DNA between the polylinker cloning site and the beginning of the *hdc* gene. This upstream region contains a putative ribosome binding site (GAGG) (Shine & Dalgarno, 1975) nine bases upstream from the AUG initiation codon. The termination codon is TAA followed by 49 nucleotides of *Morganella* DNA, part of which is an inverted repeat (Vaaler et al., 1986).

Optimal Induction Conditions. Cells made permeable with toluene and deoxycholate were assayed for HisDCase activity to test induction conditions. IPTG at 0.5 mM induced enzyme activity maximally, and pyridoxal (5 μg/L) increased enzyme activity a further 10%. Histidine proved not to be necessary for maximal activity; we added it in case the intracellular pools of histidine became depleted due to decarboxylation. Ampicillin was added to replenish that destroyed during growth. Wild-type HisDCase activity increased to a maximum level 1.5 h after induction at 37 °C and then decreased with time. The level of recombinant enzyme was also found to depend on aeration; smaller culture volumes contained more activity per milliliter than larger volumes.

Regulation of the Overproduction of HisDCase. Cultures of *E. coli* GM1 harboring the plasmid without insert do not decarboxylate histidine (Table III), demonstrating that the host does not contain a HisDCase and that no other decarboxylase is present that interferes with our assay. The activity seen in the uninduced cultures carrying pGV37 (gene correctly oriented) and pGV36 (gene backward) suggests that some transcription is occurring from an as yet unrecognized *Morganella* promoter since the same amount of activity is seen in both cases. The induced level of GM1/pGV37 indicates that optimal expression of HisDCase is under the control of the *lac* promoter. The lowered level of activity with induced GM1/pGV36 compared to the uninduced level suggests that since transcription is being forced in the opposite direction to the *hdc* gene, less HisDCase can be made. The comparatively low activities of the *Morganella* cultures indicate the advantage of having the *hdc* gene cloned.

Purification and Characterization of Recombinant HisDCase. The protocol (Table IV) for purification of recombinant HisDCase from *E. coli* GM1/pGV37 is simpler than that used for purifying the enzyme from *M. morganii* (Tanase et al., 1985) and yields pure enzyme in fewer manipulations and less time. The specific activity of ~150 μmol/(min·mg) usually obtained is twice that reported by Tanase et al. (1985). We attribute the higher activity to the presence of (hydroxypropyl)cellulose during both isolation and assays, and to the more efficient, less time-consuming purification scheme possible with the overproduced product. The 12.8-fold purification indicates that HisDCase comprised 7.8% of the total cell protein in this culture. The amino acid analysis of the recombinant HisDCase (tryptophan was not determined) and the NH_2-terminal sequence of 20 amino acids were identical with those previously reported for the enzyme from *Morganella* (Vaaler et al., 1986). The first sequence cycle yielded PTH-threonine and PTH-dehydrothreonine, but no PTH-methionine, indicating that the formylmethionine is properly removed in *E. coli*. The pure recombinant HisDCase migrated coincident with the enzyme from *Morganella* on native and denaturing polyacrylamide gels, and the two proteins showed complete cross-reactivity on Ouchterlony double-diffusion analysis with rabbit antisera to the *Morganella* enzyme. We conclude that cloned and wild-type enzymes are identical.

Some Site-Directed Mutant Enzymes Form Inclusion Bodies When Induced at 37 °C. The mutant proteins K232A, S322A, S322T, and H231R were the first to be constructed, and upon harvesting the induced cultures, we found the bacteria to be elongated and to contain inclusion bodies, i.e., insoluble aggregates of protein that appear refractile under phase-contrast microscopy. Similar inclusion bodies have been reported previously in bacteria upon overproducing various proteins including human insulin (Williams et al., 1982), prochymosin (Kawaguchi et al., 1984; Schoemaker et al., 1985), bovine growth hormone (Schoner et al., 1985), and β-lactamase and alkaline phosphatase (Georgiou et al., 1986); published photographs of such inclusion bodies resemble closely those we find in our cultures. The aggregates consisted of mutant HisDCase as demonstrated by SDS–PAGE on the 12000*g* pellet and supernatant fractions. When extracted at pH 7.5 in HEPES buffer, the S322T mutant protein was purified as insoluble material and would dissolve only in 8 M urea or 6 M guanidine hydrochloride. This could be a helpful purification step if the enzyme can be renatured. Preliminary renaturation attempts with denatured wild-type enzyme gave a 15% yield of activity.

We transformed our expression plasmids into the other host strains listed in Table I. Inclusion bodies were seen in all cultures after induction; in RB791 containing pGV37, even the wild-type enzyme aggregated into such bodies. Given longer periods of induction, the wild-type protein also aggre-

Table IV: Purification of Recombinant Histidine Decarboxylase

step	volume (mL)	total act. (units)	total protein (mg)	sp act. (units/mg)	yield (%)	purification (*x*-fold)
extraction[a]	61	17700	1723	10.3	100	1
Polymin P supernatant	58	17400	942	18.5	98	1.8
$(NH_4)_2SO_4$ ppt and dialysis	41	14500	676	21.4	82	2.1
aminohexyl-Sepharose	27	11200	120	93.0	63	9.0
DEAE-cellulose	30	8100	70.5	115	46	11.2
Sephacryl S-300	32	4600	35.2	132 (175)[b]	26	12.8

[a] From 3 L of culture, 10.6 g wet weight. [b] Calculated by using a value of $A^{1\%}_{278nm}$ = 14.5 (Tanase et al., 1985).

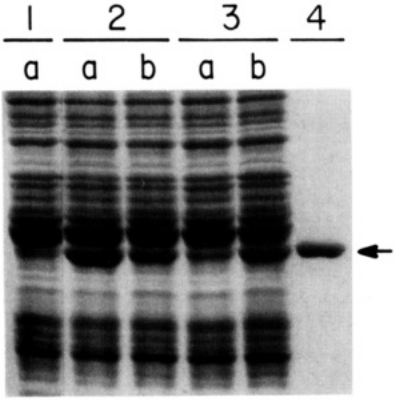

FIGURE 1: Comparison of the yield of soluble enzyme under different induction conditions. Lane 1, *E. coli* GM1/puc9 control culture; lanes 2a and 2b, GM1/pGV37 expressing wild-type HisDCase; lanes 3a and 3b, GM1/pGV40 expressing S322A HisDCase; lane 4, the arrow indicates the position of HisDCase. Cultures (150 mL) were induced at 37 °C for 1.5 h (a lanes) or at 25 °C for 4 h (b lanes). Total HisDCase from each culture is as follows: lane 2a, 7.4 mg; lane 2b, 3.6 mg; lane 3a, 0.6 mg; lane 3b, 5.8 mg. The band intensity coincident with HisDCase in lane 1a was subtracted from the HisDCase band intensities for quantitation.

Table V: Effects of Induction Temperature on the Solubility of HisDCases

	soluble HisDCase (% of total)[a] at			soluble HisDCase (% of total)[a] at	
mutation	37 °C[b]	25 °C[c]	mutation	37 °C[b]	25 °C[c]
wild-type	73	100	M233I	94	99
S229A	50	94	C240A	7	49
S229C	64	90	C240S	3	68
H231R	0[d]	60	S322A	7	96
H231F	4	73	S322T	0[d]	4
H231Q	99	100	C329A	67	100
H231N	98	97	C329S	17	100
K232A	ND[e]	100			

[a] HisDCase was determined by video area densitometry of HisDCase in the soluble (the 100000*g* supernatant) and insoluble fractions after treatment with SDS and polyacrylamide gel electrophoresis. [b] Induction at 37 °C was for 1.5 h. [c] Induction at 25 °C was for 4.0 h. [d] The solubility of these bands was judged by eye. [e] ND, not determined.

gates in the GM1 strain but to a far lesser extent than in RB791. Inclusion body formation could result from overproduction of the protein, improper folding during synthesis, or other factors. In an attempt to minimize this aggregation, we chose to synthesize protein at a slower rate by inducing the mutant cultures at 25 °C rather than 37 °C. Less wild-type enzyme is made at the lower temperature, but the 25 °C induction allows substantial production of S322A enzyme (Figure 1) and, most importantly, the enzyme is soluble. At 37 °C, the solubility of the mutant proteins ranges from 0 to 99%; all of them are equally or more soluble when induced at the lower temperature (Table V). With six of the mutant enzymes, the difference is substantial, and the 25 °C induction allows soluble protein to be isolated. Wild-type enzyme, however, is obtained in best yield when induced at 37 °C; very little enzyme is synthesized at 25 °C. Table VI lists the specific activity and the yield of mutant enzymes induced at low temperature. There is great variability in the amount of mutant enzyme produced which does not correlate with the activity of the mutant.

Replacement of Conserved Residues Evaluates Their Essentiality for Structure/Function (Table VI). Replacement of Ser-229 with Ala or Cys allowed ~7% of wild-type activity, and Gln replacing His-231 also allowed ~12% activity.

Table VI: Specific Activity and Yield of Soluble HisDCases When Induced at 25 °C for 4 h

mutation	sp act.[a] [μmol/(min·mg)]	yield[b] (mg)	mutation	sp act.[a] [μmol/(min·mg)]	yield[b] (mg)
wild-type	127.0	10[c]	M233I	38.0	123
S229A	10.0	80	C240A	96.0	47
S229C	8.0	123	C240S	68.0	37
H231R	0	58	S322A	41.0	25
H231F	0	103	S322T	1.4	2
H231Q	15.0	80	C329A	2.4	33
H231N	0.2	143	C329S	34.0	23
K232A	0	109			

[a] HisDCase present in the 100000*g* supernatant was determined as described in Table V. [b] "Yield" represents the soluble enzyme obtained from 1.5 L of culture. [c] Wild-type HisDCase is obtained in best yield by inducing at 37 °C for 1.5 h.

H231N is barely active while Phe or Arg in that position eliminates activity. The K232A mutant enzyme was inactive using the assay as described. HisDCases K232A and S322A were purified to homogeneity by the procedure described for the cloned wild-type enzyme. The purified K232A protein bound 0.92 nmol of PLP/nmol of subunit compared to 1.07 nmol of PLP/nmol of subunit for the wild-type enzyme, both determined by the phenylhydrazine assay. The K_d for histidine for the K232A protein was determined by spectrophotometric titration at 412 nm to be 1.1 mM. Lys-232 thus appears essential for catalysis by this enzyme. The specific activity of the purified S322A enzyme is 72 μmol/(min·mg), and its K_m is 2.4 mM compared to 1.1 mM for the recombinant wild-type enzyme. V_{max}/K_m ratios indicate that S322A is about 25% as efficient as wild-type in decarboxylating histidine. A Q_{12} value, defined as the specific activity at 37 °C divided by the specific activity at 25 °C, was determined to be 2.07 for wild-type and 2.04 for S322A. These data indicate that the S322A protein is not denatured at 37 °C so temperature lability does not explain inclusion body formation in cells induced at 37 °C. S322T is the only mutant protein that remains insoluble when induced at 25 °C. The enzymes with replacements at Met-233, Cys-240, and Cys-329 are all active enzymes (Table VI); we shall study their stability to oxidation in an attempt to find a HisDCase that forms crystals that are stable upon exposure to air.

Discussion

Overproduction of the PLP-dependent HisDCase from *Morganella* in *E. coli* not only allows large amounts of the enzyme to be obtained comparatively quickly and easily but also avoids the extremely unpleasant odor associated with *Morganella* cultures. The success of the lowered temperature of induction for synthesizing soluble mutant proteins is consistent with the observation that inclusion bodies frequently result when expression systems are induced by a temperature increase. In three cases (Schoner et al., 1985; O'Hare et al., 1987; Piatak et al., 1988), aggregated protein was found at a higher temperature of growth or induction (37 or 42 °C) but not at a lower growth temperature. Inclusion body formation by overproduced proteins seems to be idiosyncratic. Since soluble mutant enzymes were obtained by induction at 25 °C in this work, it is possible that with the slower biosynthesis of protein, the folding of the nascent polypeptide chain occurs more slowly and, therefore, more accurately more of the time.

There is a correlation between the yield of mutant protein and whether the mutant codon is regarded as strongly or weakly expressed in *E. coli*. We used the DNASTAR program (DNASTAR, Inc., Madison, WI) to delineate codon strength

Table VII: Occurrence of the Ser-X-His-Lys Sequence in PLP Enzymes[a]

PLP enzyme	source	sequence	ref
Histidine decarboxylase	M. morganii	S G H K	Vaaler et al., 1986
Arginine decarboxylase	E. coli	S T H K	Boeker & Snell, 1972
Lysine decarboxylase	E. coli	S T H K	Sabo & Fisher, 1974
" "	Hafnia alvei	S T H K[b]	Fecker et al., 1986
Glutamate decarboxylase	E. coli	S G H K	Strausbauch & Fischer, 1970
Tryptophan decarboxylase	C. roseus	S P H K[c]	De Luca et al., 1989
Dopa decarboxylase	Drosophila	N P H K	Eveleth et al., 1986
" "	Pig kidney	N P H K	Bossa et al., 1977
Glutamate decarboxylase	Feline brain	N P H K[c]	Kobayashi et al., 1987
Ornithine decarboxylase	E. coli	- V H K	Applebaum et al., 1975
Glycine decarboxylase (P- protein)	Chicken liver	N L H K	Fujiwara et al., 1987
Tryptophan synthase	E. coli	G A H K	Fluri et al., 1971
" "	P. putida	G A H K	Maurer & Crawford, 1971
" "	S. cerevisiae	G S H K	Zalkin & Yanofsky, 1982
" "	Neurospora	G S H K	Pratt & DeMoss, 1988
" "	S. typhimurium	G A H K	Crawford et al., 1980
Serine hydroxymethyltransferase	E. coli	T T H K	Plamann et al., 1983
" " "	Rabbit liver cytosol	T T H K	Barra et al., 1983
" " "	Rabbit liver mito	T T H K	"
Aspartate aminotransferase	Pig mito	S Y A K	Kagamiyama et al., 1977
" "	Pig cytosol	S F S K	Kondo et al., 1984
" "	Chicken mito	S Y A K	Graf - Hausner et al., 1983
" "	Chicken cytosol	S F S K	Shlyapnikov et al., 1979
" "	Rat mito	S Y A K	Huynh et al., 1980
" "	Human cytosol	S F S K	Teranishi et al., 1978
" "	Human mito	S Y A K	Martini et al., 1985
" "	E. coli	S Y S K	Kondo et al., 1984
Alanine racemase	S. typh. DadB	S V V K	Galakatos et al., 1986
Tryptophanase	E. coli	S A K K	Kagamiyama et al., 1972
Alanine aminotransferase	Pig heart	S V S K	Tanase et al., 1979
Glycogen phosphorylase	S. cerevisiae	S N M K	Lerch & Fischer, 1975
" "	Potato	S N M K	Nakano et al., 1978
" "	Rabbit muscle	G N M K	Titani et al., 1977
D - Serine dehydratase	E. coli	G S I K	Schiltz & Schmitt, 1981
Alanine racemase	S. typh. Alr	A V V K	Galakatos et al., 1986
Threonine dehydratase - degradative	E. coli	G S F K	Datta et al., 1987
" " - synthetic	E. coli	H S F K	Lawther et al., 1987

[a] The terminal lysine (K) in the sequence shown binds PLP. [b] From homology with the sequence of lysine decarboxylase from *E. coli*. [c] From homology with the sequence of DOPA decarboxylase from pig kidney.

and found that the four mutant codons that are considered strongly expressed in *E. coli* (TGC for C in S229C, TTC for F in H231F, AAC for N in H231N, and ATC for I in M233I) gave an average of 137 mg of total HisDCase (the sum of soluble and insoluble fractions) from 1.5 L of culture but four codons that are weakly expressed (GCC for A in S229A, in C240A, in S322A, and in C329A; CGC for R in H231R; TCC for S in C240S and in C329S; and ACC for T in S322T) gave an average yield of 58 mg of total HisDCase with eight mutants. This 2.4-fold difference in level of expression warrants the use of strongly expressed codons when overproducing site-specific mutant proteins in *E. coli*. We found no correlation between codon usage and inclusion body formation.

In all PLP-dependent enzymes, PLP is bound covalently to the ϵ-amino group of a lysine residue in a Schiff base linkage. Replacement of this lysine with alanine (Malcolm & Kirsch, 1985) or with arginine (Kuramitsu et al., 1987; J. Kirsch, personal communication) in aspartate aminotransferase did not prevent PLP binding, but did result in mutant enzymes lacking activity. This finding is consistent with the view that this lysine residue catalyzes the key step, the tautomerization of the external aldimine to the ketimine intermediate, in enzymatic transamination (Malcolm & Kirsch, 1985). In HisDCase, similarly, the absence of the lysine side chain in K232A does not prevent PLP binding: PLP is present in a 1:1 ratio with subunit in the isolated protein. The modified enzyme also binds histidine: its K_d of 1.1 mM is identical with K_m values (1.1 and 1.3 mM, respectively) reported by Guirard and Snell (1987) and by Tanase et al. (1985) for the wild-type enzyme. The mutant enzyme nonetheless lacks activity, suggesting that this lysine residue plays an essential role, perhaps as a proton donor, in catalysis of decarboxylation.

Currently known sequences on the NH_2-terminal side of the lysine residue that carries the P-Pxd group in various PLP enzymes are listed in Table VII. The Ser-X-His-(P-Pxd)Lys sequence is conserved in 6 decarboxylases; 13 additional enzymes possess the histidine and another 13 possess the serine in the -1 and -3 positions, respectively, relative to (P-Pxd)Lys. This conservation of sequence suggests a defined role for the histidine and serine residues for optimal enzyme structure/function. From the crystal structure of tryptophan synthase, Hyde et al. (1988) suggest that the equivalent histidine is too distant to be involved in catalysis but may play a role in coenzyme binding by partially neutralizing the charge on the phosphate group. A His-228 $\rightarrow$ Asn replacement in serine hydroxymethyltransferase (Hopkins & Schirch, 1986) yielded a mutant enzyme with a K_{cat} 25% that of wild-type, a K_m or K_d value for the substrate 2–10 times that of the wild-type enzyme, and lower thermal stability when bound to substrate. The histidine is not essential for catalysis, and the authors suggest its role is to hydrogen bond or form a salt bridge to substrate or PLP. Lowe et al. (1985) suggest that since the side-chain amide groups of asparagine and glutamine can be superimposed on the Π- and τ-nitrogens, respectively, of the imidazole group of histidine, those amino acid replacements can determine which nitrogen of the imidazole is involved in hydrogen bonding. We describe here replacement of His-231 in HisDCase with Asn, Gln, Arg, and Phe. H231N is barely active while H231Q has significant enzymatic activity (12% of wild-type). These results and Lowe's hypothesis suggest that the τ-nitrogen on the imidazole ring participates in hydrogen bonding. H231F may be an appropriate control for the volume of the imidazole side chain, and the inactivity of this mutant enzyme supports an electrostatic or hydrogen-bonding role for the histidine. H231R is inactive, possibly due to the steric bulk of the side chain.

In the crystal structure of mitochondrial aspartate aminotransferase, Ser-255 is spatially adjacent to the (P-Pxd)Lys-258 and is associated with the coenzyme phosphate (Ford et al., 1980; Kirsch et al., 1984). When the equivalent serine (Ser-229) of HisDCase is replaced with alanine or cysteine, the mutant enzymes are about 7% as active as wild-type. Therefore, Ser-229 is not essential for catalysis but is necessary for optimal structure/function, through its contribution to conformation. Previous studies showed that Ser-322 of this HisDCase was destroyed during suicide inactivation by α-(fluoromethyl)histidine (Hayashi et al., 1986). The activity of S322A demonstrates, however, that this serine is not catalytically essential. Characterization of this enzyme will be reported separately (Bhattacharjee et al., unpublished results).

A mutation of the Met-222 residue next to the catalytic site Ser-221 in subtilisin (Estell et al., 1985) decreases the sensitivity of this enzyme to oxidative changes. Met-233 in HisDCase is positioned next to the essential Lys-232 and may be oxidatively labile. We therefore prepared mutant protein M233I and also C240A,S and C329A,S which also lack a potential oxidatively labile site. All of these mutant enzymes show substantial activity, demonstrating that none of the three residues is essential. They also show varying solubilities and are promising candidates for forming crystals which, unlike the wild-type enzyme, are stable upon exposure to air and more suitable for structural investigations.

Mutational alterations in PLP enzymes can alter activity through changes in coenzyme binding as well as changes in the features common to all enzymes (substrate binding, subunit structure, proper juxtaposition of side chain residues, etc.). Evaluation of these several aspects of the mutational changes described above requires purified proteins, and is planned after purification is achieved.

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Lysine-60 in the Regulatory Chain of *Escherichia coli* Aspartate Transcarbamoylase Is Important for the Discrimination between CTP and ATP†

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ABSTRACT: Lysine-60 in the regulatory chain of aspartate transcarbamoylase has been changed to an alanine by site-specific mutagenesis. The resulting enzyme exhibits activity and homotropic cooperativity identical with those of the wild-type enzyme. The substrate concentration at half the maximal observed specific activity decreases from 13.3 mM for the wild-type enzyme to 9.6 mM for the mutant enzyme. ATP activates the mutant enzyme to the same extent that it does the wild-type enzyme, but the concentration of ATP required to reach half of the maximal activation is reduced approximately 5-fold for the mutant enzyme. CTP at a concentration of 10 mM does not inhibit the mutant enzyme, while under the same conditions CTP at concentrations less than 1 mM will inhibit the wild-type enzyme to the maximal extent. Higher concentrations of CTP result in some inhibition of the mutant enzyme that may be due either to heterotropic effects at the regulatory site or to competitive binding at the active site. UTP alone or in the presence of CTP has no effect on the mutant enzyme. Kinetic competition experiments indicate that CTP is still able to displace ATP from the regulatory sites of the mutant enzyme. Binding measurements by equilibrium dialysis were used to estimate a lower limit on the dissociation constant for CTP binding to the mutant enzyme ($>1 \times 10^{-3}$ M). Equilibrium competition binding experiments between ATP and CTP verified that CTP still can bind to the regulatory site of the enzyme. For the mutant enzyme, CTP affinity is reduced approximately 100-fold, while ATP affinity is increased by 5-fold. These data imply that lysine-60 in the regulatory chain of aspartate transcarbamoylase is partially or perhaps fully responsible for the enhanced binding of CTP over ATP to the wild-type enzyme and is partially responsible for the discrimination between these nucleotides.

Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the first reaction of the pyrimidine biosynthesis pathway, the carbamoylation of the amino group of aspartate by carbamoyl phosphate. In *Escherichia coli*, this enzyme is subject to activation by ATP and feedback inhibition by CTP and UTP, the end products of the pathway (Gerhart & Pardee, 1962, 1963; Wild et al., 1989; Yates & Pardee, 1956). The holoenzyme,[1] composed of two trimeric catalytic subunits and three dimeric regulatory subunits, exhibits homotropic cooperative interactions for aspartate binding. The catalytic subunit, which carries the active sites and is insensitive to ATP, CTP, and UTP, shows Michaelis–Menten kinetics. The regulatory subunit exhibits no catalytic activity but binds the allosteric effectors. At pH 7.0, the combination of CTP and UTP will

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[1] Abbreviations: T and R states, tight and relaxed states of the enzyme having low and high affinity, respectively, for the substance; C–R, interface between the catalytic and regulatory subunits of aspartate transcarbamoylase; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; holoenzyme, entire aspartate transcarbamoylase molecule composed of two catalytic subunits and three regulatory subunits.

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