Rat liver phenylalanine hydroxylase was expressed in both *Escherichia coli* and the *Spodoptera frugiperda* insect cell line, Sf9. Recombinant enzyme from *E. coli* was inactive and contained less than 0.1 iron atom/subunit. In contrast, recombinant enzyme expressed in Sf9 cells using a baculovirus vector was active and identical in several properties to phenylalanine hydroxylase from rat liver: the *Kₘ* for 6-methyltetrahydroppterin was 39 μM (compared with 35 μM for the rat liver enzyme), 1 atom of iron was "associated" per enzyme subunit, and electron paramagnetic resonance spectra showed that iron was distributed within two distinct environments. Putative iron-binding sites of phenylalanine hydroxylase were studied by mutating either histidine 284 or 289 to serine and expressing these mutant enzymes (PAH-H284S and PAH-H289S) in Sf9 cells. Mutants were expressed at levels similar to wild-type PAH, but contained ≤0.1 iron/subunit and were inactive. Thus, both His²⁸⁴ and His²⁸⁹ apparently are required for iron binding and hydroxylation activity.

Rat liver phenylalanine hydroxylase (EC 1.14.16.1) (PAH)¹ catalyzes the hydroxylation of phenylalanine to tyrosine in the presence of a tetrahydropterin and molecular oxygen. Physiologically, this serves to initiate detoxification of high levels of phenylalanine, and in humans, genetic deficiencies in PAH result in phenylketonuria (Shiman, 1985; Goodman, 1979). The native form of PAH is a tetramer (Fisher et al., 1972; Shiman et al., 1979) comprised of identical subunits of molecular weight 50,000 (Dahl and Mercer, 1986; Fisher et al., 1972). Ferrrous iron is required for hydroxylation to tyrosine (Fisher et al., 1972; Gottschall et al., 1982; Shiman and Jefferson, 1982) and 1 non-heme ferric iron occurs/50,000-dalton subunit (Fisher et al., 1972; Gottschall et al., 1982). During PAH activation, this iron undergoes an obligatory reduction by the pterin cofactor (Marota and Shiman, 1984; Wallick et al., 1984). Although each subunit contains only 1 iron atom, at least two different environments for the iron are detected by electron paramagnetic resonance (Bloom et al., 1986; Wallick et al., 1984). Iron binding at one of these environments gives rise to an active enzyme species, whereas population of the second environment is associated with an inactive form of enzyme. Both environments are populated in PAH from crude homogenates and are not interconvertible in the purified enzyme (Bloom et al., 1986).

Based on these observations, several models have been proposed for the role of iron in hydroxylation by PAH (Dix and Benkovic, 1988). However, its exact role in the reaction mechanism has yet to be determined, and there is little structural information regarding sites that mediate iron binding. Results from resonance Raman spectroscopy of catechol complexes of PAH (and model compounds) suggest that the iron site resembles a tripod with two imidazoles and one carboxylate as iron ligands (Cox et al., 1988). EPR spectra of the enzyme suggest that these ligands are either oxygen, nitrogen, or a combination of both, and the EPR value of *D* = 1 cm⁻¹ for the "active" iron environment (Bloom et al., 1986) is consistent with a non-sulfur ligand environment (Blumberg and Peisach, 1973; Peisach et al., 1971).

Attempts to identify iron-binding sites in PAH and to study the role of iron in enzyme mechanisms require a system for the expression of large quantities of the enzyme containing iron liganded in an active form. Such an expression system would enable studies of iron environments through site-directed mutagenesis and would potentially allow for the production of an enzyme containing ⁵⁷Fe (and possibly other transition metals) for Mössbauer studies. The cDNA encoding for PAH has previously been cloned (Dahl and Mercer, 1986). Using this cDNA, we presently have expressed recombinant PAH in *Escherichia coli* and in insect Sf9 cells using a baculovirus vector. In initial mutagenic studies, two histidines, His³⁸⁴ and His³⁸⁹, were chosen as candidates for the iron-binding ligands and were mutated independently to serine. This was based on the work of Cox et al. (1988), which suggests that at least 2 imidazoles are ligands for the iron. Also, the region between amino acids 263 and 289 has been implicated as being part of the pterin-binding sites (Jennings et al., 1991). Four histidines occur in this domain, and the reduction of iron by the pterin cofactor suggests that the iron and pterin may be within close proximity of each other. Histidines at residues 284 and 289 specifically were selected because they occur within a region that is highly conserved among all

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mammalian pterin-dependent hydroxylases (Dahl and Mercer, 1986; Grenrett et al., 1987; Grimå et al., 1985; Kwok et al., 1985) and are the only conserved histidines in PAH from Chromobacterium violaceum (Onishi et al., 1991). Thus, the present report describes the expression of active rat liver phenylalanine hydroxylase in insect Sf9 cells, the characterization of the kinetic and physical properties of the purified enzyme, and analyses of the distribution of iron between two binding environments. We also describe the expression of inactive rat liver PAH in E. coli and the physical properties of this purified recombinant enzyme. In addition, properties of two rat liver PAH mutants, H284S and H289S (as expressed in Sf9 cells), are assessed, and possible roles for His\textsuperscript{436} and His\textsuperscript{437} in iron binding are considered. 

**EXPERIMENTAL PROCEDURES**

**Materials—Restriction endonucleases and recombinant enzymes** were purchased from New England Biolabs, Boehringer Mannheim, or GIBCO/BRL. Polymerase chain reactions were performed using the GeneAmp Kit from Perkin-Elmer Cetus Instruments. PCR primers for pUC19 were a generous gift of Dr. J. Stewart. Sequence DNA sequencing supplies were from U. S. Biochemical Corp., and \(^{[35S]}\)dATP was from Du Pont-New England Nuclear. Spodoptera frugiperda Sf9 cells, wild-type AcNPV baculovirus, wild-type AcNPV with the unique EcoRI site of pVL1393 (Webb and Summers, 1990). Orientation of insertion was verified by restriction with BglII, BalI, PstI and EcoRV, and PvuII. In the construction of PAH mutants, a plasmid pRPH3 was the generous gift of Drs. Mercer, Dahl, and Cotton, the Royal Children's Hospital, Melbourne, Australia. Sheep anti-rat PAH polyclonal antibodies were the generous gift of Dr. R. Shiman, Hershey Medical School. Genius DNA-labeling reagents were from Boehringer Mannheim. All other materials were of the highest commercial quality and were used without further purification.

**Construction of Recombinant: Transfer Vectors—Standard DNA manipulations were performed as described in Sambrook et al. (1989). The recombinant transfer vector was constructed as follows. A 1.9-kilobase EcoRI cDNA fragment containing the coding sequence for rat liver PAH (Dahl and Mercer, 1986) was isolated from pRPH3 and cloned into the unique EcoRI of pVL1393 (Webb and Summers, 1990). Orientation of insertion was verified by restriction with BglII, EcoRV, and PvuII. In the construction of PAH mutants, a PstI cohesive site was used. The PAH cDNA insert of the polylinker region of pRPH3 was removed by digestion with HindIII and BamHI, blunt-ending with Klenow, and religation, yielding the plasmid ΔPfRPH3. A 640-base pair PstI fragment encoding Thr\textsuperscript{403},Glu\textsuperscript{406} was then isolated from ΔPfRPH3 and ligated into the PstI site of pUC19. The resulting plasmid, pB3J1, was used as the template for subsequent mutagenesis.**

**Site-directed mutagenesis of His\textsuperscript{436} and His\textsuperscript{437} was accomplished by overlap extension using polymerase chain reaction as described by Ho et al. (1989). Flanking primers were complementary to pUC19 and were located approximately 400 base pairs from the polylinker region.** The mutagenic primers, 5'-CTGCAACTGCTGCTGCTCTGTGCTGCTCTGGT3' for H284S and 5'-AATCTTTGGAGTGTGCTTTTGGT3' for H289S, were designed as described by Higuchi (1989). Final PCR products were isolated by agarose gel electrophoresis, digested with PstI, and ligated into the unique PstI site of pUC19. The resulting mutated plasmids, pB3J2 and pB3J4, were sequenced in both directions by the dyeodeoxy chain termination method (Sanger et al., 1977). The 640-base pair PstI fragments then were isolated from pB284 or pB289 and cloned into ΔPfRPH3. The resulting plasmids were designated pHR284 and pHR289, and the orientation of the inserted cDNA was assessed by restriction mapping with BglII, BamHI, and Mael. The 1.9-kilobase EcoRI DNA cloning fragments containing the coding sequence for the mutants H284S and H289S were isolated and cloned into the unique EcoRI site of pVL1393 (Webb and Summers, 1990).

**Cell Culture and Recombinant Virus Purification—S. frugiperda cells (Sf9 cells) were maintained in monolayer or suspension cultures at 27°C in Hink's TNM-FH medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/Trimethoprim. Methods for the growth, transfection, and infection of Sf9 cells were as described by Summers and Smith (1987). Recombinant viruses were isolated by dIution and dot blot hybridization as according to Pen et al. (1989). For each construct, clonal virus was obtained following three rounds of dilutions.**

**PAH Activity Assays—PAH activity was assayed by monitoring the rate of phenylalanine hydroxylation using iron-dependent tyrosine (Shiman and Gray, 1980). One unit of enzyme activity is defined as 1 μmol of tyrosine formed/min. The apparent Km for 6-MeH\textsubscript{1}-pterin and 6,7-MeH\textsubscript{4}-pterin also were determined using this assay system.** Cell lysates were prepared by harvesting cells from 25 cm\textsuperscript{2} culture flasks (centrifugation at 1000 x g for one wash with phosphate-buffered saline), resuspending cells in 0.3 ml of cold 0.03 M Tris-HCl, pH 7.25, 0.2 M KCl, 0.02 M phenylalanine containing 100 μg/ml phenylmethy- lylsulfonfluryl fluoride, and sonicating for 10 s with a microt and power setting of 4 (Sonifier Cell Disrupter 350). Inosoluble material was removed by centrifugation, and 30 μl of the lysate was used in the above assay. Protein concentrations were measured by the Peterson modification of the Lowry procedure (Peterson, 1977) using bovine serum albumin as a standard. Protein concentrations then were corrected according to Shiman (1980).

**Expression of Recombinant PAH in E. coli—Overnight cultures of JM108/pRPH3 (Dahl and Mercer, 1986) were inoculated into 1 liter of LB media with ampicillin (50 μg/ml) (Sambrook et al., 1989). Cultures were grown at 37°C to an A\textsubscript{600} = 0.4 and induced by the addition of 0.24 g of isopropyl-β-thiogalactopyranoside (0.1 M final concentration). Bacteria were harvested 4 h after induction and resuspended in lysis buffer 0.03 M Tris-HCl, pH 7.25, 0.2 M KCl, 0.02 M phenylalanine containing 100 μg/ml phenylmethy- lylsulfon fluoride was added to a final concentration of 100 μg/ml. Cells were lysed by incubation with 1 mg/ml lysozyme for 30 min at 4°C and sonicated in 30-s bursts until the solution was no longer viscous (Ledley et al., 1987). The preparation then was centrifuged at 16,000 x g for 40 min, and enzyme in the supernatant was activated with phenylalanine at 25°C for 5 min (Gottschall et al., 1982).** The activated supernatant was then used immediately for the purification of PAH or was stored at −70°C.

**Expression of Recombinant PAH and Mutants in Sf9 Cells—Small scale cultures on expression and activity of PAH in Sf9 cells were performed in T25cm\textsuperscript{2} culture flasks with 10% serum in 5 ml of media.** The cells were infected at a multiplicity of infection of 2 plaque-forming units/cell and cultured at 27°C for 3-7 days. Both monolayer and suspension cultures of Sf9 cells were used for large scale expression of PAH. For monolayer cultures, 25-40 T75cm\textsuperscript{2} cell culture flasks were seeded with 1 x 10\textsuperscript{5} cells/flask in 10 ml of media and infected at a multiplicity of infection of 1-5 plaque-forming units/cell. Ferrous ammonium sulfate (200 μM) then was added, and at 3 days postinfection, cultures were harvested by centrifugation at 1000 x g for 20 min and washed with 30 ml of phosphate-buffered saline. Ferrous ammonium sulfate (200 μM) 30 ml of Sf9 cells were grown to a density of 6 x 10\textsuperscript{4} to 9 x 10\textsuperscript{5} cells/ml, and cells were infected at a multiplicity of infection of 1-5 plaque-forming units/cell. Ferrous ammonium sulfate was added as above. Infected cells were cultured at 27°C with constant stirring at 100 rpm. At 3 days postinfection, cells were harvested as described for monolayer cultures. Cell lysates were prepared by resuspension in 25-40 ml of lysis buffer containing 100 μg/ml phenylmethylsulfonyl fluoride, 0.5 μM leupeptin, and 0.1 μM pepstatin and sonication (3 x 2 s with a microtip and a power setting of 4). Cell debris was removed by centrifugation at 16,000 x g for 30 min at 4°C. The supernatant was then activated with phenylalanine at 25°C for 5 min (Gottschall et al., 1982). The activated lysate was either used immediately in the purification of PAH or was stored at −70°C.

**Purification of Recombinant PAH—Recombinant PAH** was purified from E. coli or S9 cells by the method of Shiman et al. (1979) as modified by Gottschall et al. (1982) and Wallick et al. (1984). The procedure was simplified by the following modifications. After applying the homogenate, the first phenyl-Sepharose column was washed with 3 volumes of homogenization buffer containing 4.8% dimethyl formamide, followed by 3 volumes of 15% glycerin (by volume), 10 mM phenylalanine, 0.01 M EDTA, 0.033% Tween 80, and 0.03 M Tris-HCl, pH 7.25 (0.1 M final concentration). Use of a second phenyl-Sepharose column was omitted, and the enzyme was eluted directly from the DE-52 column as described by Wallick et al. (1984). The purity and subunit molecular weight of the recombinant enzyme was determined by SDS-PAGE. The native enzyme is a tetramer of equal molecular weight, as indicated by gel electrophoresis on a Superose 6 HR10/30 fast protein liquid chromatography column (Pharacema LKB Biotechnology Inc.) in 0.03 M Tris-HCl, pH 7.25.
RESULTS

Expression of Rat Liver PAH in E. coli—Rat liver PAH was expressed in E. coli using the construct of Dahl and Mercer (1986). Following lysis by sonication, the majority of the enzyme remained within soluble fractions and was readily detected by SDS-PAGE and Coomassie Blue staining. However, this preparation possessed no detectable activity in the presence or absence of ferrous iron, DTT, or liver homogenate (10% v/v). To test whether exogenous iron may be required during expression to obtain an active form of PAH, ferrous ammonium sulfate was added to the growth medium at concentrations of 100 μM, 500 μM, 1 mM, and 5 mM. This modification was without effect. In order to determine whether the lack of activity possibly was due to the presence of an inhibitor in the crude lysate, PAH expressed in the presence of 5 mM iron was purified. Purification of the enzyme by hydrophobic chromatography depends on exposure of a hydrophobic domain of PAH upon phenylalanine binding. Removal of phenylalanine restores a hydrophilic conformation resulting in the elution of the enzyme from the column. Successful purification by this procedure indicated significant native structure. In addition, recombinant PAH from E. coli was shown to occur as a tetramer (data not shown). Incubation of recombinant PAH from E. coli in urea at concentrations from 1 to 8 M with subsequent 100-fold dilution into 0.03 M Tris containing 100 μM ferrous ammonium sulfate and 500 μM DTT likewise did not result in activation. However, an assayable amount of activity was recovered by incubating PAH in 2 or 3 M guanidine HCl, followed by a 100-fold dilution in 0.03 M Tris containing 100 μM ferrous ammonium sulfate, 5 mM DTT, and 50 μg/ml catalase. The activated enzyme had a specific activity of ≥0.35 unit/mg. Increased concentrations of guanidine HCl decreased recovered activity, whereas increasing concentrations of iron, DTT, or catalase had no effect on recovery.

Expression of Rat Liver PAH in SF9 Cells—Expression of recombinant PAH in SF9 cells as mediated by baculovirus infection occurred at appreciably high levels for all seven recombinant viruses isolated (A7, B7, B8, D4, E5, F1, and H7). Based on Coomassie Blue staining of SDS gels, PAH comprised approximately 40% of total protein in cell extracts (Fig. 1). Lysates from infected cells had 0.12 unit/ml of PAH activity, whereas lysates from uninfected cells or cells infected with wild-type AcNPV had activities of ≤0.01 unit/ml. Furthermore, the addition of exogenous iron to culture media during expression greatly increased the activity of PAH, with a maximum effect being observed at 200 μM iron (Fig. 2). Increased iron concentrations did not affect detectable activity found in uninfected SF9 cells, and at iron concentrations of ≥500 μM, both cell viability and PAH expression were diminished. Iron could be added effectively at any time prior to 60 h postinfection, whereas addition at subsequent intervals resulted in decreased activity in crude lysates.

Purification and Characterization of Recombinant Rat Liver PAH—PAH was purified from infected SF9 cells grown either as suspension cultures or in monolayers. The procedure previously developed for the purification of native rat liver PAH was used (Gottschall et al., 1982; Shiman et al., 1979; Wallick et al., 1985). However, due to overproduction of PAH in SF9 cells, only limited column washes were necessary, and chromatography on a second phenyl-Sepharose column was not required. Approximately 80% of the initial activity was recovered, and purity was ≥95%.

Table I shows the typical yields of PAH obtained from infected SF9 cells. The amount of PAH protein obtained per g of wet cell weight was similar for four independent cultures. The low yield of activity in sample B is due to the omission of protease inhibitors in lysis buffer for this sample. Per g of wet cell weight, the amount of activity in the crude lysate grown in monolayers (26.8 units/g) was almost 2-fold greater than for lysates from suspension cultures (13.7 units/g). This difference also was reflected in the specific activities of PAH purified from these preparations. Purified recombinant PAH also reacted with antibodies raised against PAH from rat liver, as shown by Western blot analysis (data not shown) with no detectable cross-reactivity with either SF9 cell lysates or with lysates from cells infected with wild-type AcNPV. N-terminal analysis of purified recombinant enzyme gave the 5 amino acid sequence as Ala'-Ala'-Val'-Val'-Leu', as predicted from the cDNA sequence for PAH (Dahl and Mercer, 1986) and the N-terminal analysis of rat PAH (Iwaki et al., 1985).

With regard to enzymatic properties, recombinant PAH also had similar apparent Km values for both 6-MeH4-pterin (43 ± 4 versus 38 μM) and 6,7-MeH4-pterin (81 ± 4 versus 90 μM) compared with PAH from rat liver. Different preparations of PAH from rat liver can vary significantly in their specific activities, ranging from 3–14 units/mg with a mean
Iron Ligand Sites of Recombinant Phenylalanine Hydroxylase

FIG. 2. Effect of exogenous iron on the activity of rat liver PAH expressed in Sf9 cells. Cells were infected, harvested, lysed, and assayed as described under "Experimental Procedures." The cells were either infected with the recombinant PAH baculovirus (●) or not infected (○).

**TABLE I**

Purification of recombinant PAH

| Culture volume | Cell weight | Activity* | Yield | PAH* |
|----------------|------------|-----------|-------|------|
| ml             | g          | units     | %     | mg   |
| A              | 2 × 500b   | 6.6       | 78.0  | 90   | 15.1 |
| B              | 2 × 500b   | 6.0       | 120.2 | 65.1 | 54   | 15.9 |
| C              | 500b       | 2.9       | 30.6  | 25.2 | 82   | 5.6  |
| D              | 40 × 10b   | 4.0       | 107.1 | 84.5 | 79   | 12.0 |

* Determined as described under "Experimental Procedures."

**TABLE II**

Iron content of H284S and H289S

| Sample          | Fe/subunit* |
|-----------------|-------------|
| Wild-type PAH   | 0.85-1.07   |
| H284S           | <0.1        |
| H289S           | <0.1        |

* Determined by atomic absorption as described under "Experimental Procedures."

specific activity of 7–9 units/mg. In contrast, three different preparations of enzyme from infected SF9 cells (suspension cultures) gave specific activities of 4.2–4.9 units/mg. The conditions under which the cells are grown may also be significant in determining the specific activity, since the specific activity of PAH purified from infected monolayers (7.2 units/mg) was significantly higher than that from suspension cultures (4.6 units/mg).

Iron Content of Recombinant Rat Liver PAH—Recombinant PAH prepared from SF9 cells contained approximately 1.0 Fe/50,000-dalton subunit, as determined by atomic absorption (Table II). The EPR spectrum of this recombinant PAH contains features common to that obtained for native rat liver PAH (Bloom et al., 1986) (Fig. 3). Specifically, the resting enzyme has signals at \( g_{eff} = 6.7-5.4 \) and 4.3. The signal at \( g_{eff} = 6.7-5.4 \) is consistent with high spin Fe³⁺, whereas the signal at \( g_{eff} = 4.3 \) is associated with a form of PAH that is incapable of turnover (Bloom et al., 1986; Wallick et al., 1984). The ratio of the magnitudes of the signals at \( g_{eff} = 6.7-5.4 \) to the signal at \( g_{eff} = 4.3 \) (Fig. 3A) is consistent with a specific activity of approximately 5 units/mg.² The enzyme preparation used to generate this spectrum had a specific activity of 4.9 units/mg. The EPR spectrum in Fig. 3B is for an enzyme preparation with a specific activity of 7.0 units/mg. The signal \( g_{eff} = 4.3 \) is reduced, as compared with the signal at \( g_{eff} = 6.7-5.4 \). This is in accordance with the data reported for native PAH, where the intensity of the signal at \( g_{eff} = 4.3 \) is inversely proportional to the specific activity of the enzyme (Bloom et al., 1986; Wallick et al., 1984).

Mutagenesis of PAH at His²⁸⁴ and His²⁸⁹—In order to test whether residues His²⁸⁴ and/or His²⁹⁴ of PAH contribute to iron binding and activity, these residues were mutated independently to serine. Recombinant baculovirus vectors encoding these mutant PAHs then were prepared (D8, D12, and G6 for H284S and D8, G2, and G4 for H289S), and mutant enzymes were expressed in SF9 cells as described above. Expression of soluble protein was assessed by SDS-PAGE of cell lysates for each construct. The results for H284S are shown in Fig. 4. Similar results were obtained with H289S (not shown). For each mutant, levels of expression were comparable with wild-type. However, both H284S and H289S were more susceptible to proteolysis. As shown in Fig. 4, a major proteolytic product of approximately 35,000 daltons is observed in crude lysates. The identity of this product as PAH was confirmed by Western blot analysis (data not shown). Notably, an active 35,000-dalton fragment also can be pro-
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Table III
Activity of H284S and H289S in Sf9 cell lysates

| Sample* | Activity* | units/ml |
|---------|-----------|----------|
| Sf9 cells | 0.010 |          |
| Wild-type AcNPV | 0.004 |          |
| Wild-type PAH | 2.563 |          |
| H284S | 0.024 |          |
| D8 | 0.016 |          |
| G6 | 0.022 |          |
| H289S | 0.014 |          |
| D8 | 0.029 |          |
| G2 | 0.031 |          |

* All samples were cultured in the presence of 100 μM iron.
* Cells were cultured, harvested, lysed, and assayed as described under "Experimental Procedures."

Attempts to express rat liver PAH in E. coli resulted in the production of an inactive form of enzyme. Purification of this inactive PAH by hydrophobic chromatography through specific elution by phenylalanine, however, inferred retention of native structure. Also, the purified enzyme from E. coli occurs as a tetramer and bound antibodies prepared against rat liver PAH. However, this recombinant enzyme was shown to contain less than 0.1 Fe/50,000-dalton subunit. Rat liver PAH requires iron for activity, with a maximal activity being obtained with 1 Fe/50,000-dalton subunit (Gottschall et al., 1982; Shiman and Jefferson, 1982). Thus, the low amount of iron present in the recombinant enzyme from E. coli is sufficient to account for inactivity. The addition of exogenous iron to culture media or purified enzyme did not increase its iron content.

Attempts to activate recombinant PAH from E. coli preparations also were unsuccessful. PAH in crude rat liver can be activated in the presence of Fe²⁺ and DTT (Gottschall et al., 1982; Shiman and Jefferson, 1982). However, addition of Fe²⁺ and DTT to lysates had no effect on PAH activity. It also has been suggested that an alternate factor in the crude homogenates from rat liver may mediate PAH activation (Shiman and Jefferson, 1982). Additions of Fe²⁺, DTT, and crude rat liver homogenates likewise were without effect. Treatment of recombinant PAH in denaturants such as urea and guanidine HCl, followed by dilution in the presence of iron, DTT, and catalase, detectably increased enzyme activity. However, the specific activity of this PAH was only 3% of reported maximum activity.

Other pterin-dependent hydroxylases have been expressed in E. coli. Human PAH was expressed in E. coli, and as...
amino acid sequences from rat PAH, human PAH, rat tyrosine hydroxylase (TH), rabbit tryptophan hydroxylase (TRPH), and C. violaeceum PAH (C.v.PAH). Stars indicate the His superscript 1 and His superscript 2 in the rat PAH sequence.

determined from the crude lysate, it had the same $K_m$ and $V_{max}$ for various pterin substrates as enzyme from human liver (Ledley et al., 1987). The iron content was not determined for recombinant human PAH, but the presence of activity suggests that iron is present. Mouse (Ichikawa et al., 1991), rat (Wang et al., 1991), and human (Haavik et al., 1991) forms of tyrosine hydroxylase have also been expressed in E. coli and can be activated by the addition of iron to the assay medium. These results interestingly suggest that the incorporation of iron to rat liver PAH may require a factor or factors not present in E. coli.

The baculovirus expression system was used to express active recombinant rat liver PAH in Sf9 insect cell cultures. This expression system provides many of the protein modification, processing, and transport systems that occur in higher eukaryotic cells (Luckow and Summers, 1988). Recently, human PAH$^3$ and both rat (Fitzpatrick et al., 1990) and human (Ginsn et al., 1988) tyrosine hydroxylases also have been expressed using recombinant baculoviruses. Rat liver PAH presently is shown to be expressed at high levels in Sf9 cells, with the level of protein expression comparable with that of the polyhedrin protein, which accounts for at least 50% of the total protein detected by SDS-PAGE (Luckow and Summers, 1988). Significant levels of rat liver PAH activity were found in Sf9 cell lysates, and activity increased with the addition of exogenous iron to culture media. Purification of PAH from insect cells also was facilitated by the absence of high amounts of lipids and lipoproteins that occur in rat liver. The amount of purified enzyme obtained from 6 g of insect cells is comparable with the amount obtained from 10 rat livers (100–150 g).

Recombinant PAH, as purified from Sf9 cells, occurs as a tetramer and binds 0.85–1.07 Fe/subunit. Also, as for PAH from rat liver, iron binds to this recombinant PAH in two distinct micro-environments, as characterized by the EPR spectra of the native enzyme, with signals at $g_{tet} = 6.7$–5.4 and 4.3 (Bloom et al., 1986; Wallick et al., 1984). Wallick et al. (1984) have shown that the distribution of iron between the two environments is reflected by specific activity. Consistent with this observation, EPR spectra of recombinant PAH from Sf9 cells show decreased intensity of the signal at $g_{tet} = 4.3$, with increases in specific activity. The presence of two iron environments in the recombinant enzyme raises interesting questions about their role in the regulation of PAH activity in vivo. For example, in humans, increases in concentrations of phenylalanine to toxic levels might induce increases in the specific activity of PAH. By comparison, however, increased concentrations of phenylalanine in Sf9 cell culture media did not affect the specific activity of the recombinant PAH.

Compared with PAH from rat liver, the distribution of iron in recombinant PAH from Sf9 cells is "highly" reproducible, suggesting that additional factors also may affect activity. Oxygen, for example, is often limiting in Sf9 cell suspension cultures (Webb and Summers, 1990), and specific activities of PAH from these cultures uniformly were lower than activities for PAH isolated from Sf9 monolayers. Thus, oxygen per se might contribute to PAH activation.

Recombinant rat liver PAH mutants H284S and H289S were constructed in an attempt to identify iron-binding sites. Previous work by Cox et al. (1988) suggested that 2 histidines and 1 carboxylate are iron ligands in PAH. EPR studies of rat liver PAH also support the presence of nitrogen, or oxygen. Bloom et al. (1986) calculated a $D$ value of 1 cm$^{-1}$ for iron bound in the environment associated with active enzyme. By comparison, Blumberg and Peisach (1973) have shown that ferric iron centers with sulfur ligands usually have $D$ values of $\pm 1.5$ cm$^{-1}$, and that the value for $D$ decreases as the amount of nitrogen or oxygen ligands is increased. For example, rubredoxin with 4 sulfur ligands has a $D$ value of 1.68 (Peisach et al., 1971), whereas the non-heme iron center of nitidimo-noxin, which contains nitrogen and oxygen ligands, has a $D$ value of 0.9 (Twilfer et al., 1981). An absence of iron-sulfur charge transfers in PAH also argues against sulfur as a ligand (Cox et al., 1988).

Rat liver PAH contains 11 histidines in its primary structure (Dahl and Mercer, 1986). Of these 11 histidines, only 5 (His$^{145}$, His$^{230}$, His$^{270}$, His$^{284}$, and His$^{289}$) are conserved in rat PAH (Dahl and Mercer, 1986), human PAH (Kwok et al., 1985), rat tyrosine hydroxylase (Grima et al., 1985), and rabbit tryptophan hydroxylase (Grenett et al., 1987). Among these, histidines 270, 284, and 289 occur within a region of high homology among the mammalian hydroxylases (Fig. 5). Also, the iron environments of PAH and tyrosine hydroxylase are similar in that iron in the resting state of the enzyme is Fe$^{3+}$, which must be reduced to Fe$^{2+}$ for hydroxylation to occur (Dix et al., 1987; Fitzpatrick, 1989; Haavik et al., 1988). In addition, the EPR spectrum of tyrosine hydroxylase is similar to PAH with a signal at $g_{tet} = 5$–7 (Haavik et al., 1988), and tyrosine hydroxylase is specific in its requirement for iron (Fitzpatrick, 1989). Based on these observations, mammalian pterin-dependent hydroxylases thus are predicted to have similar, if not identical, iron-binding sites.

Mutation of either His$^{230}$ or His$^{289}$ of PAH to serine is shown to inactivate catalysis of phenylalanine hydroxylation. Each mutant nonetheless appears to retain structural features similar to wild-type enzyme. Each displays reversible phenylalanine-dependent binding to phenyl-Sepharose, and each occurs as a tetramer in the native state, as determined by gel permeation chromatography. Thus, the overall structure of these mutants appears to be unperturbed. Therefore, the lack of activity is likely attributable to the absence of bound iron in these purified mutants. Mutant enzymes were expressed in insect cell cultures under conditions that produce active wild-type PAH with approximately 1 Fe/subunit, yet H284S and H289S contain less than 0.1 Fe/subunit. A comparison of model systems with and without an imidazole ligand deletion suggest that the $K_D$ of iron will decrease at least 2–3 orders of magnitude (Hancock and Martell, 1989). Since the mutation of one of two histidine ligands should result in the loss of binding of most of the iron, our results are in accord with the involvement of both His$^{230}$ and His$^{289}$ in iron binding by rat liver PAH. Without further structural information, it is
not possible to assess to what extent the microenvironment around His284 and His289 has been altered by the mutations, and it also is formally possible that this environment is perturbed to an extent that the iron is unable to bind despite retention of alternate ligands.

A role for paired histidines as putative non-heme iron ligands has also recently been defined by site-directed mutagenesis of human 5-lipoxygenase (Nguyen et al., 1991; Zhang et al., 1992). Lipoxygenases contain non-heme iron, and a direct correlation exists between iron content and specific activity (Percival, 1991). Results from EPR spectroscopy and x-ray adsorption studies suggest this iron has 6 ligands, 4 of which are imidazoles (Navaratnam et al., 1988). When histidines 368 and 373 were replaced by serine, lysine, or cysteine, inactivation resulted (Nguyen et al., 1991; Zhang et al., 1992).

Perhaps notably, the 4-amino acid separation of the paired histidine sequence (His~~~X-~X-His~~~) is commonly distributed in PAH and the sequence mutated is His284-X-285-His286.

As mentioned above, iron in PAH binds in two different environments (Bloom et al., 1987; Wallick et al., 1984). Mutation of either His284 or His289 to serine affects binding in both environments. The EPR spectrum of the wild-type enzyme suggests that iron is either bound in a distorted tetragonal geometry (active form) or a rhombic geometry (inactive form) (Bloom et al., 1986; Wallick et al., 1984). However, it has not been determined whether the two environments contain iron coordinated to common amino acids. The results presented here suggest that iron bound in either environment has His284 and His289 as common ligands. The sequence identified by Jennings et al. (1991) as the pterin cofactor-binding site of PAH includes His284 and His289. A monoclonal anti-idiotypic antibody that mimics the binding of the pterin cofactor (Jennings and Cottrell, 1990) was used to identify this sequence (residue 263 to residue 289). Reduction of enzyme-bound iron by pterin (Marota and Shiman, 1984; Wallick et al., 1984) also suggests that the iron and pterin may bind in close proximity. Thus, general co-localization of iron- and pterin-binding sites was anticipated.

Naturally occurring mutations in the human PAH gene recently have been examined by in vitro expression analysis (Eisensmith and Woo, 1991, 1992). Among mutations located between residues 250 and 300, all are associated with severe forms of phenylketonuria. The EPR spectrum of the wild-type enzyme suggests that iron is either bound in a distorted tetragonal geometry (active form) or a rhombic geometry (inactive form) (Bloom et al., 1987; Wallick et al., 1984). However, it has not been determined whether the two environments contain iron coordinated to common amino acids. The results presented here suggest that iron bound in either environment has His284 and His289 as common ligands. The sequence identified by Jennings et al. (1991) as the pterin cofactor-binding site of PAH includes His284 and His289. A monoclonal anti-idiotypic antibody that mimics the binding of the pterin cofactor (Jennings and Cottrell, 1990) was used to identify this sequence (residue 263 to residue 289). Reduction of enzyme-bound iron by pterin (Marota and Shiman, 1984; Wallick et al., 1984) also suggests that the iron and pterin may bind in close proximity. Thus, general co-localization of iron- and pterin-binding sites was anticipated.

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