Isolation and Properties of a New, Soluble, Hemoprotein (H-450) from Pig Liver†

In-Cheol Kim and William C. Deal, Jr.*

ABSTRACT: A new soluble hemoprotein, designated as H-450, has been purified from pig liver. The absolute absorption spectrum of H-450 shows maxima at 550 and 428 nm. The dithionite-reduced H-450 has absorption peaks at 572, 540, and 450 nm; the unique Soret band at 450 nm is the basis for our tentative designation of this new hemoprotein as H-450 (hemoprotein 450). The spectrum of dithionite-reduced H-450 at 77 K gives two α peaks (571 and 566 nm), three β peaks (546, 537, and 529 nm), and a Soret band at 449 nm. The prosthetic group of H-450 has been identified as protoheme IX. Gel electrophoresis experiments show that H-450 is composed of two nonidentical subunits, α and β (mol wts = 61 000 and 45 000). H-450 contains 1 mol of heme/$\alpha\beta$ dimer of 106 000 molecular weight. Preliminary sedimentation equilibrium experiments suggest a minimum molecular weight of 218 000 for the native protein. This corresponds to a tetramer, $\alpha_2\beta_2$, containing two heme groups. H-450 is not reduced by reduced nicotinamide adenine dinucleotide (NADH), NADH phosphate, ascorbate, or ferrocyanide. Neither reduced nor oxidized H-450 binds CO, 1 mM cyanide, or 1 mM azide. Dithionite-reduced H-450 is autoxidizable. The molar extinction coefficient of native H-450 is 261×10^3 at 280 nm and 263×10^3 at 428 nm. The purification procedure involves homogenization, high-speed centrifugation, ammonium sulfate fractionation, diethylaminoethylcellulose chromatography, density gradient centrifugation, a calcium phosphate gel step, and a second density gradient centrifugation. The procedure yields approximately 2 mg of purified protein from 750 g of pig liver.

Hemoproteins, which contain iron porphyrin as a prosthetic group, are widely distributed in all living organisms and are involved in various important metabolic functions. Hemoproteins may be grouped into the following three main categories on the basis of their functions: (1) oxygen and carbon dioxide carriers, such as hemoglobin, (2) catalysts, such as catalase, and (3) electron carriers, such as cytochromes. In general, most cytochrome hemoproteins are in the insoluble fraction and most noncytochrome hemoproteins are in the soluble fraction; however, it has been reported that b_5-type cytochromes occur in the soluble fraction in pig kidney (Magnum et al., 1970) and in human erythrocytes (Passon et al., 1972).

Recently, during our work on purification of fatty acid synthetase from pig liver (Kim and Deal, 1976a), we discovered a new, soluble hemoprotein; we tentatively designated this protein as hemoprotein-450 (H-450), because of its unusual spectral properties. In this paper, we report the discovery, isolation, and characterization of this new hemoprotein.

Materials and Methods

Chemicals. DEAE[1]-cellulose was obtained from Schleicher and Schuell, Inc. We obtained bovine serum albumin, aldolase, and heart cytochrome *c* from Sigma Chemical Co.; catalase and trypsin from Worthington Biochemical Corp.; and glutamate dehydrogenase from Boehringer Mannheim Corp.

Livers. Pig livers were obtained from a local slaughter house within 1 h after slaughter and were frozen in dry ice. The livers were stored frozen at −40 °C.

Pig Blood. Pig blood was obtained from the Department of Animal Husbandry, Michigan State University, East Lansing, Mich.

Enzyme Assays. Enzyme assays were carried out at 23 °C. The decrease in absorbance at 340 nm was monitored with a Gilford 240 recording spectrophotometer.

Decalone reduction, a model partial reaction of fatty acid synthetase (Dutler et al., 1971), was assayed using (9*R*,9*S*)-*trans*-1-decalone and NADPH as substrates (Kim and Deal, 1976b).

We measured catalase and peroxidase according to Chance and Mahly (1955); tryptophan dioxygenase according to Schutz and Feigelson (1972) and Yamamoto and Hayaish (1970); cytochrome oxidase according to Wharton and Tzagoloff (1967); NADH- and NADPH-cytochrome *c* reductases according to Kim and Beattie (1973); and NADH- and NADPH-ferricyanide reductase according to Mackler (1967).

Sucrose Density Gradient Centrifugation. Sucrose density gradient centrifugation was performed according to the method of Martin and Ames (1961) with a Beckman SW 27 swinging bucket rotor in a Beckman Model L3-50 preparative ultracentrifuge at 20 °C. Solutions containing linear gradients of 5–20% sucrose were prepared in cellulose nitrate tubes using an Isco Gradient Former (Model 570). The volume per tube was 37 ml. Volumes of 1 or 1.5 ml of protein were layered on the top of the gradient solution in each tube (1 × 3.5 in.).

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received June 16, 1976.* Supported in part by grants from the National Institute of Arthritis, Metabolism and Digestive Diseases (Grant AM-15345), the National Cancer Institute (Grant CA-14017), and the Michigan State University Agricultural Experiment Station (Hatch 932; Publication 7429). A preliminary report of this work has been given (Kim and Deal, 1976c).

[1] Abbreviations used are: DR, *trans*-1-decalone reductase; FAS, fatty acid synthetase; PE, 0.2 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA; TE, 0.05 M Tris-HCl buffer, pH 7.4, containing 1 mM EDTA; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide; NADPH, NADH phosphate.

Centrifugation was performed at 27 000 rpm (131 000g) at 20 °C for 8–16 h as indicated.[2] The gradients were fractionated and analyzed at 280 nm using an Isco automatic density gradient fractionator (Model 183) with an Isco Model UA-5 absorbance monitor. The fractionation was carried out with a flow rate of 3 ml/min and fractions of 1.5 ml were collected.

Polyacrylamide Gel Electrophoresis. Electrophoresis of the native protein was conducted in 5% polyacrylamide gels at pH 8.6 according to the procedure of Davis (1964). Sodium dodecyl sulfate gel electrophoresis was carried out according to the method of Weber and Osborn (1969). Staining of the gels in the presence of benzidine and hydrogen peroxide was performed according to Welton and Aust (1974).

Absorption and Difference Spectra at Room Temperature. The absolute spectra were determined with a Cary 15 spectrophotometer. For difference spectra of dithionite-reduced minus oxidized samples, a few grains of dithionite were added to the sample cuvette.

For difference spectra of CO-reduced protein minus reduced protein, both sample and reference solutions were reduced with dithionite, then the sample cuvette was bubbled with carbon monoxide gas for 10, 30, or 60 s before scanning, to see if there was any change in the difference spectra with time. In other experiments, dithionite was added after introduction of CO.

Low-Temperature Spectra of H-450. A 0.5-ml sample of an H-450 preparation containing 50% glycerol and TE buffer was placed in a vertical cylindrical cuvette and frozen to 77 K with liquid nitrogen in a cylindrical Dewar. Absorption spectra were measured, keeping the spectral bandwidth less than 0.4 nm, using a single-beam spectrophotometer on line with a small computer similar to that described by Butler and Hopkins (1970). Other conditions are according to the procedure of Davis et al. (1973). Derivative analyses of the absorption spectra were performed to assist in identifying the absorption peaks.

Pyridine Hemochrome Experiment. The nature of the heme group was determined using the alkaline pyridine hemochrome method (Falk, 1964); the solvent contained 0.1 N NaOH and 20% pyridine. Using the known value of 32.4 cm^{-1} mM^{-1} for the difference in extinction coefficients at 557 and 575 nm for dithionite-reduced minus oxidized difference spectra of known pyridine hemochrome (Omura and Sato, 1964), the heme content of H-450 was determined from the appropriate difference spectra with H-450 samples.

Sedimentation Equilibrium. The high-speed meniscus depletion sedimentation equilibrium experiment was carried out as described by Chervenka (1970). A 12-mm double-sector cell with sapphire windows and interference window holders was used. H-450 was dialyzed overnight at 4 °C against TE buffer containing 0.06 M KCl. A 0.12-ml aliquot of sample (0.7 mg/ml) was added to the right sector of the cell after 0.01 ml of fluorocarbon oil (FC-43) had been added; 0.13 ml of solvent was added to the left sector of the cell. The experiment was carried out in an An-G rotor at 9300 rpm at 25.3 °C for 24 h. The Rayleight interference pictures were taken after 24 h, with spectroscopic II-G plates. The log of the fringe displacement, log $(Y_i - Y_0)$ was plotted against the square of the distance (r^2) and the weight-average molecular weight was calculated from the slope of the line.

[2] The purer H-450 preparations show a tendency to aggregate in sucrose solutions; the H-450 peaks move faster in the later density gradient experiments then in the first one.

Results

Purification of H-450. Unless indicated otherwise, all purification steps were carried out at 4 °C and all centrifugation steps were carried out in a Sorvall RC-2B centrifuge using a GSA rotor (25 min at 11 000 rpm) for large volumes (20 000g) or an SS-34 rotor (15 min at 16 000 rpm) for small volumes (31 000g). The H-450 protein initially copurifies with fatty acid synthetase and the partial reaction decalone reductase assay for fatty acid synthetase is used to locate the fractions containing H-450, in the first parts of the purification; beginning with the gradient centrifugation step, the H-450 is concentrated enough to follow using its absorption at 428 nm or by using the absorption at 450 nm of samples reduced with dithionite.

High-Speed Supernatant Fraction. Frozen pig liver (750 g) was cut into small pieces and homogenized for 1 min at maximum speed in a Waring blender in 1800 ml of cold TE buffer.[1] The homogenate was centrifuged in a GSA rotor at 11 000 rpm for 15 min. The supernatant was further centrifuged in polycarbonate bottles at 21 000 rpm (60 000g) for 2 h using a Beckman Type 21 rotor in a Beckman Model L3-50 preparative ultracentrifuge.

Ammonium Sulfate Fractionation. The supernatant solution was brought to 0.2 saturation with saturated ammonium sulfate and centrifuged (GSA rotor); the resulting supernatant solution was brought to 0.33 saturation and centrifuged. The precipitate was collected and suspended in 1200 ml of TE buffer. Saturated ammonium sulfate was added to 0.33 saturation. The pellet obtained after centrifugation was resuspended in 100 ml of TE buffer and dialyzed overnight against 4 l. of TE buffer with one buffer change.

DEAE-Cellulose Chromatography. The dialyzed enzyme was divided into two parts and applied to two DEAE-cellulose columns (41 × 33 cm) at room temperature. A linear KCl gradient (0–0.234) in TE buffer was used for elution (3.1 l. total). The fractions that had decalone reductase activity were pooled, brought to 0.33 saturation with saturated ammonium sulfate solution, and centrifuged. The pellet was suspended in PE buffer, and dialyzed overnight against 2 l. of PE buffer.

First Gradient Centrifugation. The dialysate was used for sucrose density gradient centrifugation (16 h), as described under Materials and Methods.

The faster moving peak fractions that had decalone reductase activity were pooled and precipitated by addition of ammonium sulfate to 0.5 saturation. After centrifugation for 15 min at 16 000 rpm, the reddish pellet was suspended in a small volume of 5 mM potassium phosphate buffer, pH 7.2, and desalted on a Sephadex G-25 column (2.4 × 35 cm) with 5 mM potassium phosphate buffer, pH 7.2. This yielded a bright-yellow solution.

Calcium Phosphate Gel Step. A suspension of calcium phosphate gel was added to the solution from the previous step (2 mg of gel/mg of protein). After gently stirring for about 3 min at room temperature, this suspension was centrifuged for 7 min at 16 000 rpm. The reddish-brown pellet (calcium phosphate gel) was washed, using a Teflon-coated glass homogenizer, with 50 ml of 5 mM potassium phosphate (pH 7.2) about seven times, or until the decalone reductase activity in the supernatant after centrifugation was negligible. Then the hemoprotein was eluted by extracting the calcium phosphate gel three times with 50 ml of 0.2 M potassium phosphate buffer, pH 7.4. The three solutions of supernatant obtained after centrifugation were combined and brought to 50% ammonium sulfate saturation with saturated ammonium sulfate solution.

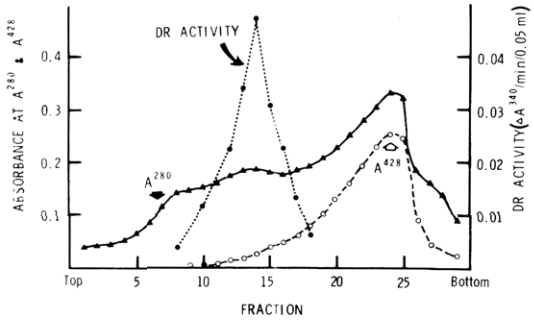

FIGURE 1: The sucrose density gradient centrifugation profile of the fraction eluted from the calcium phosphate gel. H-450 was eluted from calcium phosphate gel, precipitated at 0.5 ammonium sulfate saturation, and centrifuged. The pellet was suspended in PE buffer and dialyzed against the same buffer at 4 °C overnight. Sucrose density gradient centrifugation was performed at 27 000 rpm for 13 h at 20 °C, as described under Materials and Methods. The contaminating fatty acid synthetase was measured using a partial reaction, a decalone reductase (DR) assay. Activity is expressed as the absorbance change at 340 nm per min per 0.05 ml.

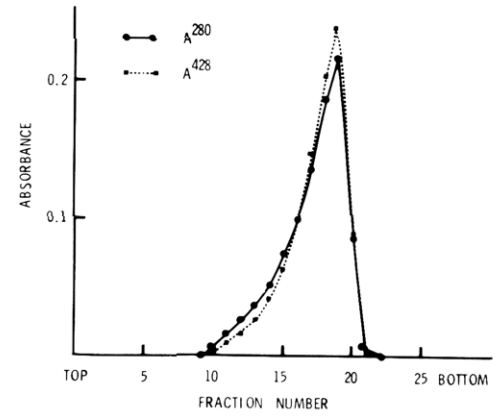

FIGURE 2: Profile of the final sucrose density gradient centrifugation step. The fractions containing H-450 from the second sucrose density gradient centrifugation (Figure 1) were pooled and precipitated with 50% ammonium sulfate saturation. The pellet was suspended in PE buffer and dialyzed at 4 °C overnight against the same buffer. The dialysate was applied to tubes containing linear 5–20% sucrose density gradients. The centrifugation was carried out at 27 000 rpm for 8 h at 20 °C.

Second and Third Gradient Centrifugation Steps. The reddish-yellow pellet obtained after centrifugation was suspended in about 1 ml of PE buffer and dialyzed against 500 ml of the same buffer overnight. The dialysate was applied to 5–20% sucrose density gradient tubes and centrifuged at 20 °C for 13 h at 27 000 rpm.

H-450 fractions were detected by absorption at 428 nm (Figure 1). Fractions free of contaminating decalone reductase were pooled and brought to 50% ammonium sulfate saturation. After centrifugation for 15 min at 16 000 rpm, the pellet was suspended in a minimal volume of PE buffer and dialyzed overnight against the same buffer (1000 ml). The dialysate was again applied to 5–20% sucrose density gradient tubes and centrifuged at 27 000 rpm for 8 h, at 20 °C (Figure 2). Fractions containing H-450 were pooled and precipitated at 0.5 saturation with saturated ammonium sulfate solution. The pellet obtained after centrifugation at 16 000 rpm for 15 min was suspended in a minimal volume of PE buffer and dialyzed against the same buffer overnight. The dialysate was centrifuged at 16 000 rpm for 15 min and the pellet was discarded. This homogeneous H-450 preparation was stored at 4 °C.

Various Aspects of the Purification Procedure. H-450 copurifies with fatty acid synthetase up to the calcium phosphate gel step where H-450, but not fatty acid synthetase, is strongly absorbed. However, some fatty acid synthetase is absorbed and appears in the H-450 eluted from the calcium phosphate gel with 0.2 M potassium phosphate, pH 7.4 (see Figure 1). Since the peaks of fatty acid synthetase and H-450 are well separated in the second[2] (Figure 1) and third (Figure 2) sucrose density gradient centrifugation steps, the contaminating synthetase is completely removed. From 750 g of frozen pig liver, about 2 mg of homogeneous H-450 was obtained. The H-450 preparation meets the criteria of purity in sucrose density gradient sedimentation velocity experiments (Figure 2), polyacrylamide electrophoresis (Figure 3A), and sedimentation equilibrium.

When purification of H-450 was attempted from the pig blood, the final preparation was colorless and therefore showed no absorption at 428 nm; hence, no H-450 was isolated from pig blood and H-450 originates from liver tissue, not hepatic blood.

Native and Subunit Structures. Electrophoresis experiments with the native H-450 at pH 8.6 (Figure 3A) gave a single protein band. In sodium dodecyl sulfate gel electrophoresis

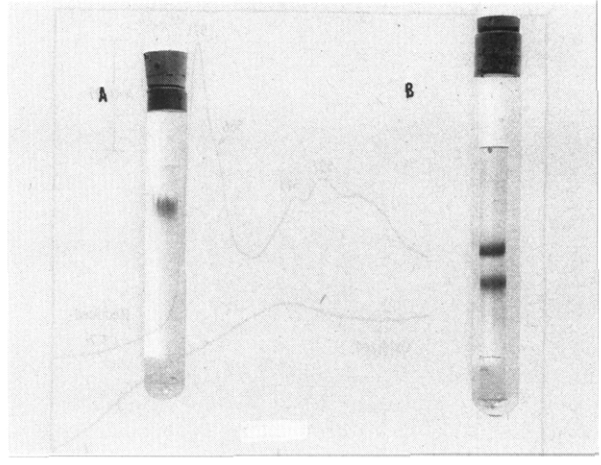

FIGURE 3: Five percent polyacrylamide gel electrophoretic patterns of H-450. (A) Standard disc electrophoresis patterns of native H-450. Electrophoresis was carried out according to Davis (1964). (B) Sodium dodecyl sulfate gel electrophoresis. The experiment was performed according to Weber and Osborn (1969).

experiments, the same preparation gave two protein bands (Figure 3B), indicating that H-450 is composed of two nonidentical subunits, present in approximately equal amounts. These subunits are designated α and β from this point on. From a standard calibration curve, the molecular weight values for the α and β subunits were found to be 61 000 and 45 000, respectively.

From a meniscus depletion sedimentation equilibrium experiment, which yielded a linear ln c vs. r^2 graph, the molecular weight of native H-450 was calculated to be 217 600. Based on this and the subunit molecular weight values, native H-450 must be an $\alpha_2\beta_2$ tetramer.

Room Temperature Spectra of H-450. As indicated in Figure 4, the untreated (oxidized) H-450 has absorption maxima at 550 and 428 nm; the α band is not well defined, but a shoulder at 590 nm was observed with the instrument on a more sensitive setting. Absorption peaks of the dithionite-reduced protein showed absorption maxima at 572, 540, and 450 nm. These properties are typical of hemoproteins, except for the unusually high wavelength (450 nm) of the Soret band.

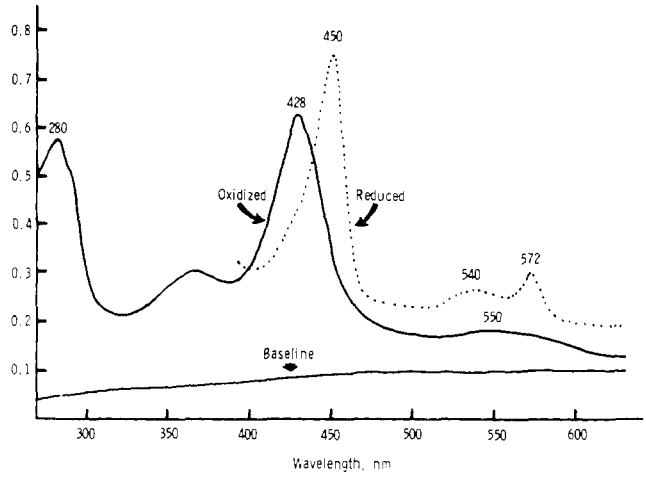

FIGURE 4: Spectra of oxidized and dithionite-reduced H-450 at room temperature. H-450 was dialyzed against PE buffer. The baseline was obtained with identical samples of H-450 in each compartment. Absolute spectra of oxidized and reduced H-450 were obtained at 23 °C as described under Materials and Methods. The concentration of H-450 was 0.45 mg/ml.

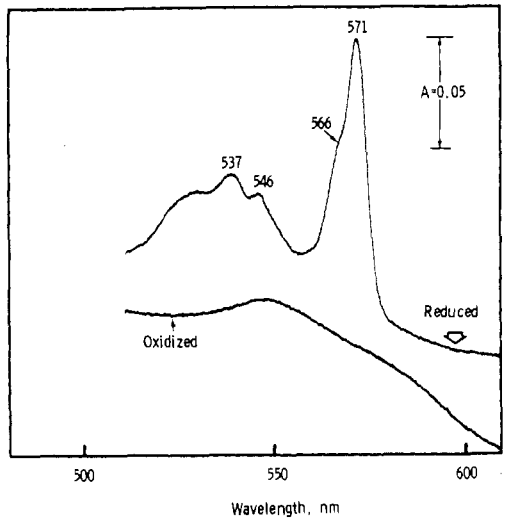

FIGURE 5: Low temperature spectra of H-450 in 50% glycerol. The upper curve is the spectrum of dithionite-reduced H-450 at 77 K. The lower curve is the spectrum of oxidized (untreated) H-450 at 77 K. The solution contained H-450 (0.23 mg/ml) in TE buffer with 50% glycerol.

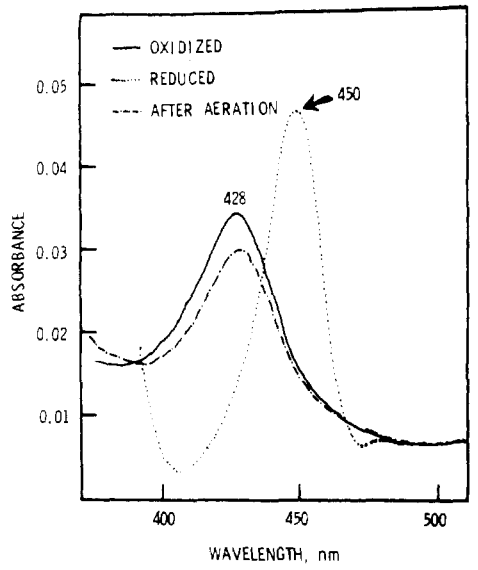

FIGURE 6: Aeration of dithionite-reduced H-450. After reduction of an H-450 preparation with a few grains of dithionite, aeration was performed for 15 s before obtaining the absolute spectrum. Spectra were obtained only in the Soret region for these studies.

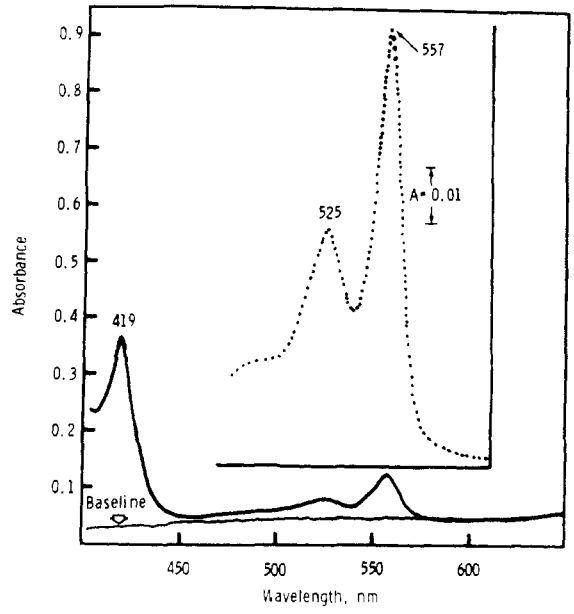

FIGURE 7: Spectrum of the pyridine hemochrome of H-450. This experiment was performed as described under Materials and Methods. The absolute spectrum of dithionite-reduced pyridine hemochrome was obtained at room temperature. The baseline was obtained using the solvent, 0.1 N NaOH–20% pyridine, in both compartments. The insert is the spectrum of the α and β region on an expanded absorbance scale. The final concentration of H-450 was 0.23 mg/ml.

This value is about 20 to 30 nm higher than the corresponding peak for most known hemoproteins.

So far, only P-450 has been reported to have a 450-nm absorption peak and that is produced with the reduced protein, but only in the presence of carbon monoxide (Omura and Sato, 1964; van der Hoeven and Coon, 1974). However, under analogous conditions, the addition of CO to our H-450 preparation did not produce any change in absorption. This indicates that H-450 is not P-450.

Low-Temperature Spectra of H-450. In 50% glycerol solutions at 77 K, dithionite-reduced H-450 gave two α peaks (571 and 566 nm), three β peaks (546, 537, and 529 nm), and a Soret band at 449 nm (Figure 5). Oxidized H-450 showed a broad β peak at 548 and a Soret band at 428 nm (not illustrated). The absorption peaks in dithionite-reduced minus oxidized difference spectra of H-450 were the same as those in dithionite-reduced spectra. It is interesting to note that the wavelengths of the α peaks of H-450 are about 10 nm longer than those of cytochrome *b* in bovine heart mitochondria (Davis et al., 1973; Hagihara et al., 1975) under comparable conditions.

Autoxidation of H-450. Native H-450 exists in the oxidized form. It was of interest to see whether dithionite-reduced H-450 could spontaneously be reoxidized as other cytochromes. Accordingly, a sample of H-450 was, first, completely reduced with dithionite and then aerated for 15 s. As shown in Figure 6, almost all of the H-450 was completely reoxidized.

Properties of the Heme Group of H-450. As shown in Figure 7, the pyridine hemochrome spectrum of H-450 gave absorption maxima at 557, 525, and 419 nm, which indicates that

TABLE I: Properties of H-450.

(1) Found in soluble fraction
(2) Absorption bands:

	α	β	Soret
Oxidized (native)		550	428 nm
Reduced	572	540	450 nm

(3) Does not bind CO, CN^-, or N_3^-
(4) Autoxidizable
(5) Structure:
Native protein: $\alpha_2\beta_2$; mol wt = 218 000
Subunit mol wts: α = 61 000; β = 45 000
(6) Contains 2.1 mol of heme/mol ($\alpha_2\beta_2$) of H-450; heme is protoporphyrin IX
(7) Molar extinction coefficients for the 218 000 mol wt tetramer:

Wavelength (nm)	550	428	280
ϵ	42.6×10^3	263×10^3	261×10^3

the prosthetic group of H-450 is protoporphyrin IX. The heme content was found to be 2.1 mol/mol of 218 000 tetramer of H-450.

To determine whether various strong field ligands might bind to the heme group, we obtained spectra of untreated (oxidized) H-450 and spectra of dithionite-reduced H-450, both in the presence and in the absence of CO, 1 mM cyanide, and 1 mM azide. None of these ligands had any effect on the spectra and we conclude that they do not bind to the heme group of H-450.

Tests for Catalytic Functions of H-450. To further characterize H-450, we tested its ability to catalyze a number of reactions. All of these tests were negative. H-450 did not exhibit any of the following activities: NADH or NADPH dehydrogenase, NADH- and NADPH-cytochrome *c* reductase, cytochrome *c* oxidase, NADH- and NADPH-ferricyanide reductase, catalase, or tryptophan dioxygenase activities. Even though peroxidases are known (Saunders et al., 1964) to occur in mammalian liver, none have been purified or characterized thus far. H-450 did not exhibit peroxidase activity either.

The properties of H-450 are summarized in Table I.

Discussion

Origin. H-450 is a newly discovered hemoprotein from pig liver with unique spectral and structural properties. Since the clarified crude extract (59 000*g* × 2 h) contains some contaminating microsomal fraction, we can not rule out that H-450 is loosely bound to microsomes and easily detached during homogenization. To the best of our knowledge, cytochrome b_5 (Spatz and Strittmatter (1971)) and P-450 (Omura and Sato, 1964; van der Hoeven and Coon, 1974) are the only cytochromes or hemoproteins definitely established as being present in the microsomal fraction. The following section will show that H-450 has many cytochrome-like properties. But the only mammalian cytochrome known to occur in the soluble fraction is cytochrome b_5; it has been reported to be in the soluble fraction in pig kidney (Mangum et al., 1970) and erythrocytes (Passon et al., 1972). Although not conclusive, the evidence, thus far, is most consistent with H-450 being a cytoplasmic protein.

Search for Function. Though H-450 initially copurifies with fatty acid synthetase, the activity of fatty acid synthetase fractions is not affected by the removal of H-450 (Kim and Deal, 1976a). We have no evidence yet which suggests any relationship between these two proteins, but this possibility has not been excluded and is receiving further study.

H-450 did not exhibit any of the following enzymatic activities: NADH- and NADPH-dependent dehydrogenase, NADH- and NADPH-dependent cytochrome *c* reductase, cytochrome *c* oxidase, NADH- and NADPH-dependent ferricyanide reductase, catalase, or tryptophan dioxygenase. It is known that most heme proteins have some catalase and peroxidase activity (Falk, 1964). However, H-450 does not possess either catalase or peroxidase activity, with hydrogen peroxide as substrate. So, it appears that H-450 is not one of the peroxidases which have been discovered in mammalian liver (Saunders et al., 1964; Hunter, 1955) but which have not yet been purified or characterized.

H-450 possesses several properties which suggest that it is a cytochrome-type hemoprotein. H-450 has a low-spin iron, based on its spectral properties (Mahler and Cordes, 1966), and this is consistent with it being a cytochrome. Most non-cytochrome hemoproteins possess high-spin iron. The properties of H-450 are generally closer to those of *b*-type cytochromes. However, as mentioned previously, the only mammalian cytochrome know to exist in the soluble fraction is cytochrome b_5.

H-450 possesses another property similar to that of certain cytochromes; samples of reduced or oxidized H-450 do not show any spectral change in solutions containing CO, 1 mM potassium cyanide, or 1 mM sodium azide, compared to solutions without these substances. Hence, as in cytochrome *c* (Paleus and Paul, 1963) and intact mitochondrial *b*-type cytochromes (Hagihara et al., 1975; Ohnishi, 1966), the fifth and sixth ligands of the heme moiety of H-450 are not accessible to these strong-field ligands. It remains to be determined whether H-450 is involved as an electron transfer carrier in an, as yet, undiscovered electron transport process in the soluble fraction in mammalian liver.

Unusual Properties of H-450. Table I lists some of the properties of H-450. A number of the properties of H-450 are unusual and, in some cases, unique. The Soret band at 450, the basis for its name, is one of the most unique properties of H-450. It should be emphasized again that this characteristic is observed with dithionite-reduced H-450 and, unlike P-450, does not require the presence of CO; in fact, CO has no effect on the spectral curve.

The structure of H-450 is also unusual. Native H-450 is a tetramer (mol wt 218 000) containing two α subunits (mol wt 61 000), two β subunits (mol wt 45 000), and two heme groups. Thus, there is only one heme group per two subunits. Most other hemoproteins and cytochromes contain one heme per subunit. However, there are some other exceptions. Both the tryptophan dioxygenase from rat liver (Schutz and Feigelson, 1972) and side chain cleavage cytochrome P-450 from bovine adrenal mitochrondria (Shikita and Hall, 1973) have only one heme per two subunits.

Another unusual feature of H-450 is that it does not bind strong field ligands, such as CO, CN^-, and azide. This indicates that the fifth and sixth ligands of the heme moiety are already tightly bound, probably by the subunits. Since there is one heme group per $\alpha\beta$ dimer, this could be accomplished either by binding at two different sites on one type of subunit, or by binding one site on each type of subunit. Cytochrome *c*, which contains only one type of subunit, is the only hemoprotein presently described which binds one heme group to two binding sites on the same subunit (Paleus and Paul, 1963).

The previous section has already described in detail another unusual property of H-450, namely, that it appears in the

soluble fraction, despite the fact that it possesses many properties of a cytochrome (especially cytochrome *b*), most of which are not found in the soluble fraction.

It may be emphasized, in conclusion, that the previously described subunit structure and ligand binding properties of H-450 are clearly unique among soluble hemoproteins. Most other soluble hemoproteins: (1) bind with strong ligands, indicating that one of the heme binding sites is open; (2) consist of homologous subunits (e.g., catalase) or analogous subunits (e.g., hemoglobin); and (3) contain one heme group per subunit.

Acknowledgment

Appreciation is expressed to the following persons for very helpful discussions regarding this work: Dr. Phillip D. Bragg, University of British Columbia; Dr. Diana S. Beattie, Mount Sinai School of Medicine, New York; Dr. Steven Aust, Michigan State University; and Dr. Donald E. Hultquist, University of Michigan. We are also indebted to Drs. K. L. Poff and K. Manabe, Michigan State University, for the low temperature spectrum experiments and discussions about them.

References

Butler, W. L., and Hopkins, D. W. (1970), *Photochem. Photobiol. 12,* 439.

Chance, B., and Mahly, A. C. (1955), *Methods Enzymol. 2,* 763, 773.

Chervenka, C. H. (1970), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Spinco Division of Beckman Instruments, Inc., p 56.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Davis, K. A., Hatefi, Y., Poff, K. L., and Butler, W. L. (1973), *Biochim. Biophys. Acta 71,* 150.

Dutler, H., Coon, M. J., Kull, A., Vogel, H., Waldvogel, G., and Prelog, V. (1971), *Eur. J. Biochem. 22,* 203.

Falk, J. E. (1964), *Porphyrins Metalloporphyrins,* 110, 181.

Greengard, O., and Feigelson, P. (1962), *J. Biol. Chem. 247,* 5327.

Hagihara, B., Sato, N., and Yamanaka, T. (1975), *Enzymes, 3rd Ed. 11,* 549.

Hunter, M. J. (1955), *Methods Enzymol. 2,* 791.

Kim, I. C., and Beattie, D. S. (1973), *Eur. J. Biochem. 36,* 509.

Kim, I. C., and Deal, Jr., W. C. (1976a), *Arch. Biochem. Biophys.* (in press).

Kim, I. C., and Deal, Jr., W. C. (1976b) (submitted for publication).

Kim, I. C., and Deal, Jr., W. C. (1976c), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1423.

Mackler, B. (1967), *Methods Enzymol. 10,* 294.

Mahler, H. R., and Cordes, E. H. (1966), Biological Chemistry, New York, N.Y., Harper and Row, p 586.

Mangum, J. H., Klingler, M. D., and North, J. A. (1970), *Biochem. Biophys. Res. Commun. 40,* 1520.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Ohnishi, K. (1966), *J. Biochem. (Tokyo) 59,* 9, 17.

Omura, T., and Sato, R. (1964), *J. Biol. Chem. 239,* 2370.

Paleus, S., and Paul, K. G. (1963), *Enzymes, 2nd Ed. 8,* 97.

Passon, P. G., Reed, D. W., and Hultquist, D. E. (1972), *Biochim. Biophys. Acta 275,* 51.

Saunders, B. G., Holmes-Siedle, A. G., and Stark, B. P. (1964), in Peroxidase, Washington, D.C., Butterworths, p 41.

Schutz, G., and Feigelson, P. (1972), *J. Biol. Chem. 247,* 5327.

Shikita, M., and Hall, P. F. (1973), *J. Biol. Chem. 248,* 5605.

Spatz, L., and Strittmatter, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1942.

van der Hoeven, T. A., and Coon, M. J. (1974), *J. Biol. Chem. 249,* 6302.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 1969.

Welton, A. F., and Aust, S. D. (1974), *Biochem. Biophys. Res. Commun. 56,* 898.

Wharton, D. C., and Tzagoloff, A. (1967), *Methods Enzymol. 10,* 245.

Yamamoto, S., and Hayaish, O. (1970), *Methods Enzymol. 17,* 434.