Characterization of Hemopexin and Its Interaction with Heme by Differential Scanning Calorimetry and Circular Dichroism†

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ABSTRACT: Hemopexin is a plasma glycoprotein that has two structural domains (I and II) and binds and transports heme particularly to liver cells. Differential scanning calorimetry (DSC) studies show that hemopexin is largely stabilized by heme, which binds exclusively to domain I. The melting temperature (T_m) of heme–hemopexin is 66.4 ± 0.7 °C as compared with 53.9 ± 0.3 °C for apohemopexin, and this T_m increase is accompanied by a 100 kcal increase in molar enthalpy. Heme stabilizes hemopexin by stabilizing domain I. This is demonstrated by the 26 °C increase in T_m from 51.9 ± 0.3 to 77.6 ± 0.6 °C and the over 3-fold increase in molar enthalpy when domain I associates with heme. A moderate change in domain I secondary structure is indicated by an increase in negative molar ellipticity at 206 nm. However, there is no net effect on the secondary structure of holo-hemopexin caused by heme binding as indicated by both far-UV circular dichroism (CD) and Fourier-transform infrared spectra. The characteristic positive ellipticity of hemopexin at 233 nm, ascribed to tryptophan residues in domain II, is dramatically increased, suggesting a change in tertiary structure for domain II of hemopexin. DSC and CD results show that isolated domain I and domain II interact both in the presence and absence of heme. Moreover, domain II destabilizes heme–domain I, which may be an important factor in facilitating heme release to the hemopexin receptor. Examination of a variety of heme analogs revealed three classes of effects on both the thermodynamic stability and the positive ellipticity of hemopexin at 233 nm: heme and cobalt–protoporphyrin increase both; iron-*meso*-tetrakis(4-sulfonatophenyl)porphine does not affect either; protoporphyrin and nickel–protoporphyrin decrease both.

Hemopexin is a serum glycoprotein which transports heme to the liver *via* a receptor-mediated process (Smith & Morgan, 1979, 1981). Hemopexin binds heme tightly ($K_d \sim 1$ pM, Hrkal *et al.*, 1974), and the interaction with heme produces characteristic changes in the absorbance, fluorescence and circular dichroism (CD)[1] spectra of hemopexin (Morgan, 1976; Morgan *et al.*, 1976; Morgan & Vickery, 1978). The heme-induced alteration in conformation associated with the change in CD is believed to be important for receptor-mediated heme transport (Smith *et al.*, 1988). Plasmin preferentially cleaves rabbit hemopexin (60 kD, 437 amino acid residues) at a single site, generating two non-disulfide-linked domains: a 35-kDa N-terminal domain I and a 25-kDa C-terminal domain II. Domain I binds 1 mol of heme/mol in a manner analogous to intact hemopexin while domain II facilitates the binding of hemopexin to its receptor (Smith & Morgan, 1984; Morgan & Smith, 1984; Morgan *et al.*, 1988). Some heme analogs such as tin–protoporphyrin (SnPP) and iron-*meso*-tetrakis-(4-sulfonatophenyl)porphine (FeTPPS) also influence the structure of the hemopexin molecule (Smith *et al.*, 1988; Morgan *et al.*, 1988b).

Major questions remain on the structural nature of the interaction of hemopexin with its ligands and on the role of conformational changes in both recognition of heme–hemopexin by the hemopexin receptor and the mechanism of heme release. The work reported here was undertaken to address some of these questions using CD and differential scanning calorimetry (DSC). The results demonstrate that hemopexin is largely stabilized by heme, that different heme analogs have different influences on the structure and stability of hemopexin, and that isolated hemopexin domains interact in the presence and absence of heme. Moreover, these studies provide further information on the accommodation of heme in the heme-binding pocket of hemopexin as well as structural information relevant to understanding binding of hemopexin to its receptor.

MATERIALS AND METHODS

Hemopexin was isolated from rabbit serum as described (Morgan & Smith, 1984; Morgan *et al.*, 1993), and the activity and purity (greater than 95%) of the isolated protein were determined from the characteristic absorbance and heme-binding properties of the molecule (Smith & Morgan, 1979; Morgan, 1976; Morgan & Muller-Eberhard, 1972) and by SDS-polyacrylamide gel electrophoresis using 4–20% acrylamide gradient gels. Rabbit hemopexin was used in this work since its interactions with heme and heme analogs are characterized more fully than those of other species of hemopexin. Protoheme, mesoheme, deuteroheme, protoporphyrin, cobalt–protoporphyrin (CoPP), SnPP, FeTPPS and nickel–protoporphyrin (NiPP) were obtained from Porphyrin Products (Logan, UT). *N*-Bromosuccinimide (NBS) was purchased from Sigma Chemical Co. and recrystallized from distilled water. NBS solutions were prepared immediately

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[1] Abbreviations: ferriprotoporphyrin IX; FeDP, ferrideuterioporphyrin IX; FeMP, ferrimesopoporphyrin IX; Fe(II)PP, ferroprotoporphyrin; PP, protoporphyrin IX; CoPP, Co-protoporphyrin IX; FeTPPS, ferri-*meso*-tetrakis(4-sulfonatophenyl)porphine; SnPP, Sn–protoporphyrin IX; NiPP, Ni–protoporphyrin IX. CD, circular dichroism; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; NBS, *N*-bromosuccinimide.

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before each experiment in 0.1 M sodium phosphate, pH 6.6. Samples were treated with NBS for 5 min at room temperature and analyzed immediately. Sodium dithionite was purchased from J. T. Baker Chemical Co. Reduced heme–hemopexin was prepared immediately before use by adding solid sodium dithionite. The state of reduced heme–hemopexin was confirmed by UV/vis spectra recorded before and after CD measurements.

Stoichiometric metalloporphyrin–hemopexin complexes were prepared as described (Smith & Morgan, 1981, 1984). Concentrations were measured spectrophotometrically using published extinction coefficients (M^{-1} cm^{-1}): 1.7×10^5 at 404 nm for protoheme and at 394 nm for mesoheme in dimethyl sulfoxide (Brown & Lantzke, 1969); 1.1×10^5 at 280 nm for apohemopexin (Seery *et al.*, 1972); and 1.5×10^5 at 392 nm for FeTPPS in 0.1 M NaCl, pH 3.3 (Gibbs *et al.*, 1980). The extinction coefficients of the other heme analogs used are as follows: 1.64×10^5 at 406 nm for SnPP in 0.5% pyridine containing 1 drop of NH_4OH/100 mL; 1.8×10^5 at 424 nm for CoPP in the mixture of 0.1 M NaOH, pyridine and water (v/v 3:10:17); 1.5×10^5 at 410 nm for NiPP in dimethyl sulfoxide; and 1.7×10^5 at 392 nm for deuteroheme in dimethyl sulfoxide (B. Burnham, Porphyrin Products, personal communication).

Calorimetric measurements were carried out on a high-resolution Microcal MC-2 differential scanning calorimeter at protein concentrations in the range 25–30 μM. The solvent was 25 mM sodium phosphate buffer, pH 7.4, and the scan rate was 60 °C/h. To analyze the reversibility of unfolded proteins, after scanning to 90% completion the samples were quickly cooled to room temperature and rescanned. All curves were corrected for the instrumental base line obtained by filling both cells with the buffer used. Integration procedures were carried out using software provided by Microcal, Inc. after normalization of the data for protein concentration and subtraction of the chemical base line, *i.e.*, the straight line connecting the initial and final temperatures of the overall transition. In the case of two-component samples, curve-fitting was performed under the assumption of two transitions in the unfolding process using software provided by Microcal, Inc. Different unfolding models were tried iteratively to vary the peak intensity to minimize the root-mean-square error until no further significant improvements were achieved in the error term.

CD spectra were recorded with a Jasco J-720 spectropolarimeter. The instrument was calibrated with an aqueous solution of (+)-10-camphorsulfonic acid (CSA) from Aldrich Chemical Co. at a concentration of 1 mg/mL in a 1-mm cell. This compound has a $\Delta\epsilon$ of 2.36 at its CD maximum of 290.5 nm, or an ellipticity of 33.5 millidegree (Chen & Yang, 1977). The concentration of the proteins were *ca.* 25 μM in 25 mM sodium phosphate buffer, pH 7.4, and cylindrical quartz cells with a 0.1-mm path length were routinely used. Spectra were usually scanned from 260 to 184 nm at a scan speed of 20 nm/min with 10 accumulations. The averaged spectra were corrected for the buffer blank and normalized for protein concentration (determined spectrophotometrically).

Lyophilized proteins were dissolved in D_2O 24 h in advance of infrared analysis. Infrared spectra were measured with a Mattson Sirius-100 Fourier transform infrared (FT-IR) spectrophotometer at room temperature using CaF_2 windows and 25-μm path length. For each spectrum, a 1000-scan interferogram was collected in single-beam mode with a 2-cm^{-1} resolution from 4000 to 500 cm^{-1}. Reference spectra were recorded under identical conditions with only the media in which the protein was dissolved in the cells. The reference spectra were then subtracted from the spectra of the protein solution.

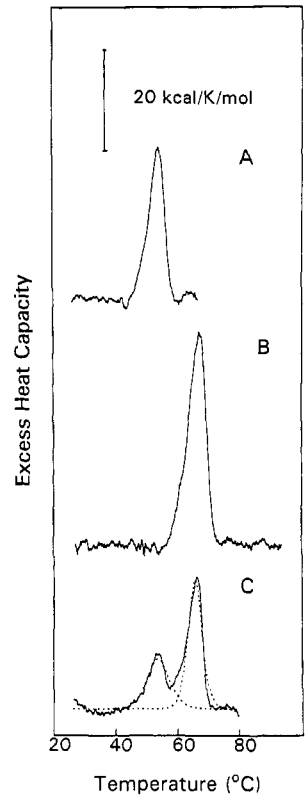

FIGURE 1: Calorimetric scans of apohemopexin, heme–hemopexin and an equimolar mixture of apo- and heme–hemopexin. Shown are (a) apohemopexin; (b) heme-hemopexin; and (c) an equimolar mixture of apo- and heme–hemopexin. The dotted lines in (c) are fitted individual peaks. The concentrations of apohemopexin and heme–hemopexin were 30 μM, respectively, in 25 mM sodium phosphate buffer, pH 7.4, and all data were normalized for concentration. The scan speed was 60 °C/h, and further details are given in Materials and Methods.

RESULTS

Hemopexin and Heme. The thermodynamic stability of hemopexin is dramatically increased upon its binding to heme as shown by DSC. The T_m of heme–hemopexin is 66.5 ± 0.6 °C as compared with 53.9 ± 0.3 °C for apohemopexin (Figure 1), and this increase in T_m is accompanied by an increase in molar enthalpy (ΔH_d) from 185 ± 7 kcal/mol for apohemopexin to 292 ± 5 kcal/mol for heme–hemopexin (Table I). The stoichiometry of heme and hemopexin is 1:1 ($K_d \sim$ pM; Hrkal *et al.*, 1974), so that a solution of hemopexin half saturated with heme contains equimolar amounts of heme–hemopexin and apohemopexin. The expected two transitions are observed in this case, corresponding to apohemopexin and heme–hemopexin with their respective T_m and ΔH_d values (Figure 1, Table I). The thermal unfolding of hemopexin and heme–hemopexin is irreversible, since no endotherm is seen on rescanning (data not shown).

Far-UV CD (Figure 2) and amide I′ FT-IR spectra (Figure 3) indicate that there is no appreciable net effect on the secondary structure of hemopexin produced by heme binding, but the characteristic positive molar ellipticity of hemopexin at 233 nm increases by 80% (Table II). This positive ellipticity at 233 nm is ascribed to tryptophan residues in domain II of hemopexin based on its abolition by chemical modification of tryptophan residues by *N*-bromosuccinimide (NBS; Figure

Table I: Parameters Obtained from DSC Scans of Hemopexin and Its Domains[a] in the Presence and Absence of Heme and Heme Analogs

sample	T_M[b] (°C)	$\Delta H_d(T_M)$[c] (kcal/mol)
hemopexin	53.9 ± 0.3	185 ● 7
hemopexin + heme	66.4 ± 0.7	292 ± 5
hemopexin + $^1/_2$heme[d]	53.9/66.4	89/146
domain I	51.9 ± 0.3	94 ± 5
domain I + heme	77.6 ± 0.6	368 ± 42
domain II	49.3 ● 0.5	140 ● 9
domain I + domain II[e]	54.2/62.4	138/105
domain I + domain II + heme[f]	55.8/74.7	233/318
hemopexin + PP[g]	53.3 ● 0.4	131 ± 10
hemopexin + CoPP[g]	70.0 ± 1.0	321 ± 16
hemopexin + FeTPPS[g]	57.4 ± 0.7	143 ± 7
hemopexin + SnPP[g]	55.1 ± 0.5	157 ± 4
hemopexin + NiPP[g]	49.2 ± 0.2	75 ± 5

[a] The concentrations of hemopexin and its domains were around 30 μM, and the buffer was 25 mM sodium phosphate, pH 7.4. [b] Observed melting temperature. [c] Calculated enthalpy change at melting temperature. [d] Equimolar mixture of hemopexin and heme–hemopexin. [e] Equimolar mixture of domain I and domain II. [f] Equimolar mixture of heme–domain I and domain II. [g] Equimolar mixture of metalloporphyrin and hemopexin.

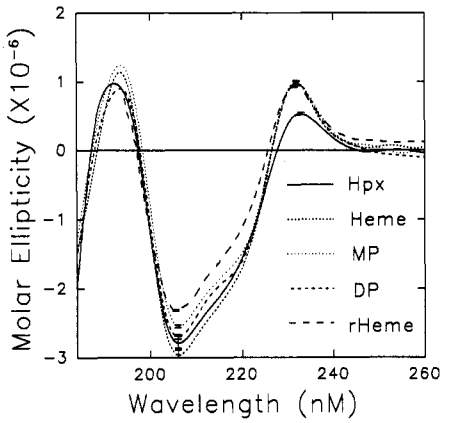

FIGURE 2: Circular dichroism spectra of hemopexin and heme–hemopexin. Circular dichroism spectra of apohemopexin (solid line), heme–hemopexin (short dashed line), FeDP–hemopexin (medium dashed line), FeMP–hemopexin (dotted line), and Fe(II)PP–hemopexin (long dashed line) were recorded at 25 °C in 25 mM sodium phosphate buffer, pH 7.4. The concentrations of the protein and the equimolar complexes were near 25 μM. Data were normalized to the exact concentration and are presented as molar ellipticity, [θ] deg cm^2 dmol^{-1}. Further details are given in Materials and Methods.

4). The reaction was performed at pH 6.6 instead of the more commonly used lower pH values since the positive ellipticity of hemopexin diminishes at pH below 6.0 (Morgan & Muller-Eberhard, 1972), and the NBS reaction can cleave peptide bonds at low pH. After an average of 3 out of 9 tryptophan residues were modified, domain II loses all ellipticity at 233 nm (Figure 4), which supports that tryptophan residues are the main contributors to the ellipticity. Since domain II does not bind heme, it is likely that changes in the environment of these tryptophan residues are transduced from domain I to cause the increase. As expected, FeDP, FeMP and Fe(II)PP have almost the same effect on hemopexin as heme, suggesting the same accommodation of these four iron-porphyrins in the binding site of hemopexin domain I.

Hemopexin Domains and Heme. Binding of heme to the 35-kDa domain I of hemopexin increases the T_m from 51.9 ± 0.3 °C to 77.6 ± 0.6 °C with an accompanying more than 3-fold increase in molar enthalpy (Figure 5a,b, Table I). Figure 6 shows the far-UV CD spectra of domain I and the heme-domain I complex. The negative molar ellipticity at 206 nm

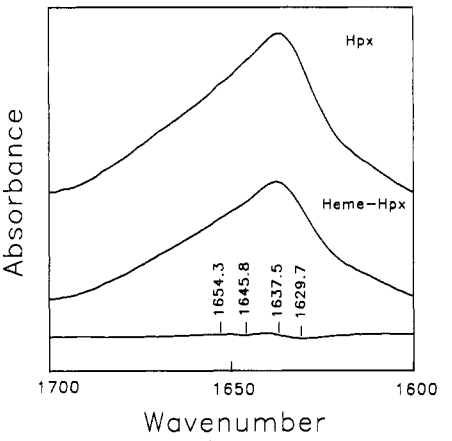

FIGURE 3: Infrared spectra of hemopexin and heme–hemopexin in the region of the amide I′ band (1700–1600 cm^{-1}). FT-IR spectra were obtained in D_2O as described under Materials and Methods. The concentrations of the protein samples were near 25 mg/mL. The difference spectrum of hemopexin and heme–hemopexin is also shown (bottom curve).

Table II: Parameters Obtained from CD Scans of Hemopexin and Its Domains in the Presence and Absence of Heme

sample	$[\theta]^a \times 10^{-5}$ at 233 nm	$[\theta] \times 10^{-6}$ at 206 nm
hemopexin	5.3 ● 0.2	−2.79 ± 0.1
domain I	−1.2 ● 0.1	−2.28 ± 0.06
domain II	6.1 ± 0.4	−0.76 ± 0.05
domain I + domain II	3.6 ± 0.3	−3.04 ± 0.04
domain I + domain II (A)[b]	4.9	−3.04
hemopexin–heme	9.8 ± 0.3	−2.96 ± 0.1
domain I–heme	−1.0 ● 0.1	−1.78 ● 0.03
domain I–heme + domain II	5.5 ± 0.4	−2.57 ± 0.08
domain I–heme + domain II (A)[b]	5.1	−2.54

[a] Ellipticities shown here were obtained in 25 mM sodium phosphate buffer, pH 7.4 and are in units of deg cm^2 dmol^{-1}. [b] Arithmetic addition of observed ellipticities of domain I, heme domain I, and domain II.

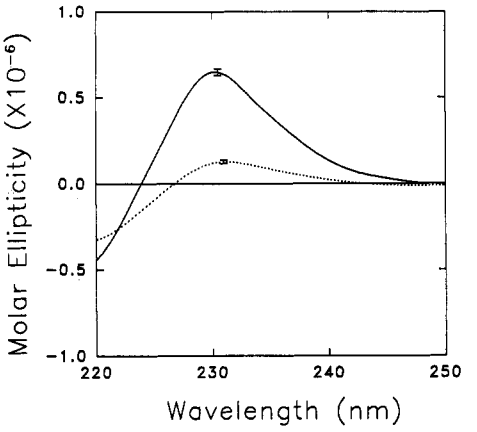

FIGURE 4: Circular dichroism spectrum of domain II of hemopexin before and after tryptophan modification by NBS. The circular dichroism spectrum of domain II before (solid line) and after (dotted line) modification were recorded at 25 °C in 120 mM sodium phosphate buffer, pH 6.6. The concentration of domain II after modification was corrected for the addition of NBS solution. Data are presented as molar ellipticity, and additional details are given in Materials and Methods.

decreases from 2.28 × 10^6 to 1.78 × 10^6 deg cm^2 dmol^{-1} (Figure 8 and Table III) which suggests moderate changes in secondary structure possibly associated with the compaction of domain I when it binds heme (Morgan *et al.*, 1988).

The results of both DSC and CD experiments clearly show that isolated domain I and domain II interact. The T_m of

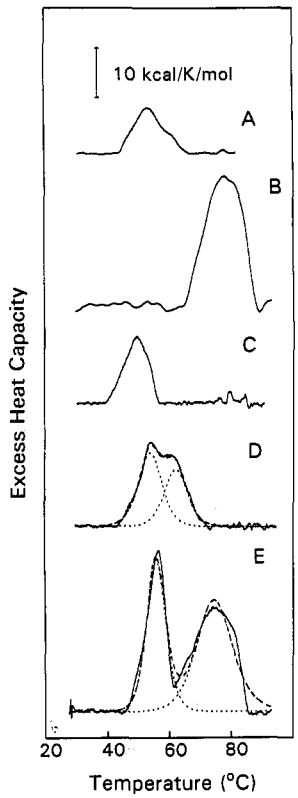

FIGURE 5: Calorimetric scans of hemopexin domains in the presence and absence of heme. The DSC profiles of hemopexin domain I (a), heme–domain I (b), domain II (c), an equimolar mixture of domain I and domain II (d), and an equimolar mixture of heme–domain I and domain II (e) were recorded in 25 mM sodium phosphate buffer, pH 7.4. The dotted lines in (d) and (e) are fitted individual peaks. The concentrations were all near 30 μM, and the scans were normalized for exact concentration in each case. The heating rate was 60 °C/h, and additional details are given in Materials and Methods.

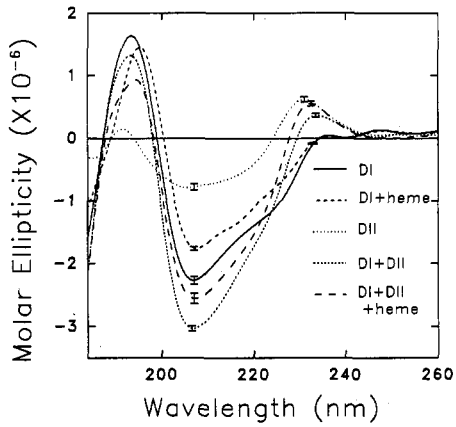

FIGURE 6: Circular dichroism spectra of hemopexin domains in the presence and absence of heme. Circular dichroism spectra of domain I (DI, solid line), heme–domain I (medium dashed line), domain II (DII, dotted line), an equimolar mixture of domain I and domain II (short dashed line), and an equimolar mixture of heme–domain I and domain II (long dahsed line) were recorded at 25 °C in 25 mM sodium phosphate buffer, pH 7.4. The concentrations were all near 25 μM, and the spectra were normalized for the exact concentration. Data are presented as molar ellipticity, and further details are given in Materials and Methods.

apo-domain I and domain II alone are 51.9 and 49.3 °C (Figure 5a,c), respectively, while an equimolar mixture (25 μM each) of the two domains displays melting temperatures of 62.4 and 54.2 °C (Figure 5d) with their respective ΔH_d values remaining unchanged. The increase in T_m for both domains demonstrates

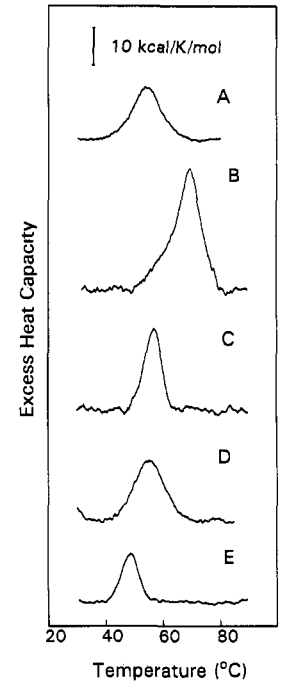

FIGURE 7: Calorimetric scans of the complexes of hemopexin with heme analogs. Equimolar mixtures of hemopexin and different heme analogs: (a) PP, (b) CoPP, (c) FeTTPS, (d) SnPP, and (e) NiPP, were prepared in 25 mM sodium phosphate, pH 7.4. The concentrations of these complexes were near 30 μM, and the data were normalized for exact concentration. The scan speed was 60 °C/h, and additional details are given in Materials and Methods.

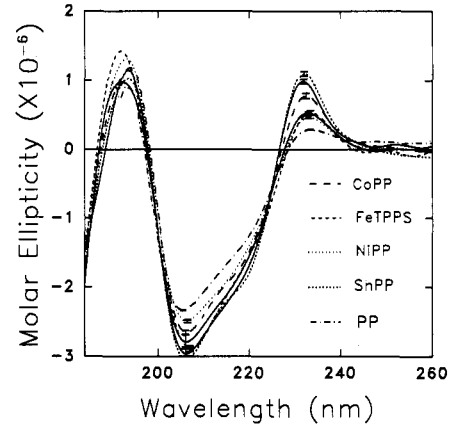

FIGURE 8: Circular dichroism spectra of the complexes of hemopexin with heme analogs. Circular dichroism spectra of CoPP–hemopexin (long dashed line), FeTPPS–hemopexin (medium dashed line), SnPP–hemopexin (short dashed line), NiPP–hemopexin (dotted line) and PP-hemopexin (dash dotted line) were recorded at 25 °C in 25 mM sodium phosphate buffer, pH 7.4. For comparison, the spectra of apohemopexin and heme–hemopexin (both solid lines) are also shown. The concentrations of all the complexes above were near 25 μM. Data were normalized to the exact concentration and are presented as molar ellipticity, and further details are given in Materials and Methods.

an interaction between them in the absence of heme. In the presence of heme, the increase in both T_m and ΔH_d (Table I) for both domains indicates not only that domain II interacts with heme–domain I complex but also that heme consistantly exerts overall stabilization effects on domain I and hemopexin.

The interaction of isolated domains was further explored by CD spectroscopy. Domain II is responsible for the characteristic positive ellipticity of hemopexin at 233 nm, and neither domain I nor heme–domain I contributes much to the ellipticity at this region (Figure 6 and Table II). Upon mixing

Table III: Parameters Obtained from CD Scans of Hemopexin in the Presence and Absence of Heme and Heme Analogs

sample	$[\theta]^a \times 10^{-5}$ at 233 nm	$[\theta] \times 10^{-6}$ at 206 nm
hemopexin	5.3 ± 0.2	−2.79 ± 0.1
hemopexin–FePP[b]	9.8 ± 0.3	−2.96 ± 0.1
hemopexin–FeDP	9.8 ± 0.2	−2.55 ± 0.02
hemopexin–FeMP	9.7 ± 0.3	−2.70 ± 0.03
hemopexin–Fe(II)PP	9.3 ± 0.1	−2.32 ± 0.01
hemopexin–CoPP	7.8 ± 0.4	−2.66 ± 0.03
hemopexin–SnPP	11.2 ± 0.3	−3.01 ± 0.03
hemopexin–FeTPPs	5.4 ± 0.3	−2.89 ± 0.04
hemopexin–NiPP	4.7 ± 0.3	−2.67 ± 0.02
hemopexin–PP	2.9 ± 0.1	−2.30 ± 0.01

[a] Molar ellipticity, deg cm^2 dmol^{-1}, is shown for each. [b] The abbreviations used are as follows: FePP, ferriprotoporphyrin IX; FeDP, ferrideuteroporphyrin IX; FeMP, ferrimesoporphyrin IX; Fe(II)PP, ferroprotoporphyrin; CoPP, cobalt–protoporphyrin IX; SnPP, tin–protoporphyrin IX; FeTPPS, ferri-*meso*-tetrakis(4-sulfonatophenyl)porphine; NiPP, nickel–protoporphyrin IX; PP, protoporphyrin.

equimolar amounts of isolated apo-domain I and domain II, the ellipticity of domain II at 233 nm decreases significantly (Figure 6 and Table II), again indicating a substantial interaction between apo-domain I and domain II. However, mixing heme–domain I with domain II restores some of the increase in ellipticity at 233 nm.

Hemopexin and Heme Analogs. Different heme analogs exert different effects on the thermostability of hemopexin (Figure 7 and Table I). CoPP has a stabilizing effect on hemopexin that is very similar to that of heme with an increase in T_m of 16 °C and an increase in ΔH_d of *ca.* 130 kcal/mol. FeTPPS and SnPP, however, do not affect the thermostability of hemopexin and exhibit about the same T_m and ΔH_d as the apoprotein. The T_m of PP–hemopexin is the same as apohemopexin with the ΔH_d being a little smaller. NiPP on the other hand destabilizes hemopexin, shifting the T_m of apohemopexin from 53.9 to 49.2 °C for the NiPP–hemopexin complex. Accompanying this is a 2.5-fold decrease in ΔH_d from 185 kcal/mol for apohemopexin to 75 kcal/mol for NiPP–hemopexin.

Figure 8 depicts the CD spectra of hemopexin complexed with the heme analogs. As shown by the far-UV spectra, no significant secondary structural alterations are involved, although subtle or compensating changes could occur and not be detected. With respect to their positive ellipticities at 233 nm, CoPP and SnPP are larger, FeTPPS is the same and NiPP and PP are smaller than the ellipticity of apohemopexin at this wavelength.

DISCUSSION

The current structural model of hemopexin consists of two domains linked by a "hinge" region where a plasmin digestion site is located. When hemopexin binds heme, the two domains interact with each other and the hinge region is no longer accessible to plasmin (Morgan *et al.*, 1988). This model is extended by the present thermodynamic and spectral studies.

Hemopexin and heme–hemopexin have enthalpies of 192 and 296 kcal/mol corresponding to 3.1 and 4.9 cal/g, respectively. These values are in the range of normal values for globular proteins with apohemopexin being lower and heme-hemopexin somewhat higher than the reported average value of 4.2 cal/g (Hu & Sturtevant, 1987). The increased enthalpy and substantial increase in T_m show that hemopexin is stabilized to an appreciable extent by heme. Heme–domain I is a very stable complex with a T_m of 77.6 °C and enthalpy of 10.5 cal/g; however, the almost 4-fold increase in enthalpy of domain I (T_m of 51.9 °C) upon binding heme is not unusual. For example, Engeseth and McMillin (1986) reported a 6-fold increase in enthalpy when azurin binds Cd^{2+}.

The DSC curves of both apohemopexin and heme-hemopexin show a distinct asymmetry (Figure 1), with the low-temperature slope being more gradual than the high-temperature slope. One possible explanation is that a small fraction of the hemopexin molecules have a different stability than the majority. However, a more likely explanation can be derived from the two-domain-structure of hemopexin. The unfolding of each of these two domains has a substantial influence on the other, but their transition states may not overlap completely. The second explanation is supported by the DSC curves of the isolated domains being more symmetric than those of apo- and heme-hemopexin (Figure 5).

The thermal unfolding of hemopexin and heme–hemopexin are apparently irreversible under the conditions used, since no endotherm is seen on rescanning (data not shown). Yet, after two scans no precipitation of either sample occurred. This can be explained by the absence of free sulfhydryl groups and high content of carbohydrate (20% by weight) of hemopexin. It has been shown that the enthalpy change associated with protein thermal unfolding primarily arises from the exposure to solvent of buried apolar groups in the protein as well as from the rupture of hydrogen bonds involved in secondary and tertiary structure formation (Privalov & Gill, 1988). The lack of any distinguishable transition state in the rescan of hemopexin is expected for a protein with an extended conformation without stable secondary or tertiary structures.

The complexes of hemopexin with heme analogs in general have broader transition states than those of apohemopexin and heme–hemopexin. Since the heme analogs differ in one or more aspects from the natural ligand, more than one type of association with hemopexin may occur with concomitant slight differences in thermostability. The broader DSC curves can be resolved into two or more individual transition states, but a detailed analysis of these observations is outside the scope of the present study.

The exact function of domain II in the heme transport process is unknown, but it has been shown that domain II aids the hemopexin and hemopexin receptor interaction (Smith & Morgan, 1984; Morgan & Smith, 1984; Morgan *et al.*, 1988). The destabilization effect of domain II on domain I, especially when domain I binds heme in the intact protein, is notable as evidenced by the changes in T_m from 77.6 and 49.3 °C for heme–domain I and domain II alone to 74.7 and 55.8 °C for the mixture (Table I). This relative destabilization may be an important factor in facilitating conformational changes in domain I that aid release of heme after hemopexin binds to its receptor, since hemopexin is not degraded but recycles intact (Smith & Morgan, 1979; Smith & Hunt, 1990).

The interaction of isolated domains in the presence and absence of heme is further shown by CD studies. The ellipticity of domain II at 233 nm decreases significantly upon mixing with an equimolar amount of apo-domain I, indicating a substantial interdomain influence. Furthermore, heme increases the ellipticity at 233 nm of domain II by binding to domain I in the intact protein and apparently also in the mixture of isolated domains. The latter is suggested by the restoration of some of the increase in ellipticity of domain II at 233 nm when heme–domain I and domain II are mixed (Table II).

The amide I′ region (1700–1600 cm^{-1}) is very sensitive to protein secondary structure, and individual bands have been assigned to α-helix (1653 cm^{-1}), β-structure (1637 cm^{-1} and others), and aperiodic structure (1645 cm^{-1}) (Byler & Susi,

1988). The difference infrared spectrum of hemopexin and heme–hemopexin shows no distinctive bands, which indicates that no appreciable net change in secondary structure of hemopexin occurs on binding heme. Moderate changes in secondary structure of domain I are observed when domain I binds heme as indicated by the decrease of the negative molar ellipticity at 206 nm in the far-UV CD spectra. This observation is further supported by the infrared spectra of apo- and heme–domain I (Wu & Morgan, manuscript in preparation). Complementary changes in secondary structure of domain II in the heme–hemopexin complex are anticipated, since no apparent net change in secondary structure of the holo-protein is seen in the presence and absence of heme.

Very few proteins have a positive ellipticity at 233 nm in their CD spectra, and certain tryptophan residues in proximity with each other have been proposed to be responsible for this ellipticity of hemopexin (Morgan & Muller-Eberhard, 1974). Rabbit hemopexin has 17 tryptophan residues, and nearly all of them are conserved among the mammalian hemopexins so far sequenced, including those from human (Takahashi *et al.*, 1985; Altruda *et al.*, 1985), rat (Nikkila *et al.*, 1991), rabbit (Morgan *et al.*, 1993) and mouse (A. Smith, personal communication). In the present study, modification of only three of nine tryptophan residues by NBS (Spande & Witkop, 1967) in domain II caused domain II to lose almost all ellipticity at 233 nm. In addition, the heme binding activity of hemopexin and domain I were lost (data not shown). However, whether tryptophan residues are in the heme binding site or whether side reactions of NBS with histidine residues occurred is not known. In any case, the conformation of the tryptophan residues in domain II is of interest, and the X-ray crystal structure of domain II is being determined (Baker *et al.*, 1993).

The magnitude of the positive ellipticity at 233 nm appears to be highly dependent on the environment of the tryptophan residues. Heme makes the whole protein more compact and thus presumably changes the environment of the tryptophan residues, resulting in an 80% increase in the molar ellipticity at 233 nm (Table III). The previous report of a 50% increase in the molar ellipticity after heme binding (Morgan & Muller-Eberhard, 1972) may well have been due to partial proteolytic digestion of hemopexin (SDS-PAGE gels were not run on older preparations) since the molar ellipticity of the mixture of heme–domain I and domain II is nearly the same as that of the apoprotein (Figure 6 & Table II).

On the basis of their effects on the positive ellipticity at 233 nm of hemopexin, three groups of metalloporphyrin-hemopexin can be distinguished (Table III): Some increase the ellipticity including ferri-proto-, -meso- and -deutero-porphyrins, ferro-PP, CoPP and SnPP; some, like FeTPPS, do not affect the ellipticity; and some, like NiPP and PP, decrease the ellipticity. The correlation between the effect of these heme analogs on the stability (T_m and ΔH) and the conformation of hemopexin (assessed by ellipticity at 233 nm) is notable. A metalloporphyrin which increases the stability of hemopexin also increases the ellipticity, and one that decreases stability decreases ellipticity. Those that do not affect the stability of hemopexin do not affect ellipticity. The ferric ion of heme is coordinated by side chains of two histidine residues (Morgan *et al.*, 1993) in the heme-binding pocket of domain I, and upon binding heme, domain I becomes both more stable (this work) and more compact (Morgan *et al.*, 1988b). The induced conformational changes in domain I may alter the interactions between domain I and domain II, thus providing a different environment for tryptophan residues in domain II, and thereby increasing the ellipticity.

In this regard, CoPP probably also coordinates with histidine residues like heme and would be expected to mimick the effects of heme. NiPP, however, appears to make domain I less stable, therefore decreasing the stability of the whole protein. The interaction of NiPP with domain I may provide yet another environment for the tryptophan residues in domain II, one that leads to a decrease in the ellipticity. This hypothesis is supported by the early studies of McLees & Caughey (1968) which showed that Ni(II) binds two axial piperidine ligands with much less affinity and lower enthalpy than iron. It can be expected that NiPP also binds axial histidines with much lower affinity than iron and is unable to stabilize hemopexin. Interestingly, PP, which cannot be coordinated by histidine residues, resembles NiPP in the sense that it also has a much lower affinity for hemopexin than heme (Morgan, 1976). The decrease of enthalpy and molar ellipticity thus appears to reflect the inability to form strong coordinate bonds between histidine and the ligand. In the case of FeTPPS, coordination of heme iron by histidine residues would tend to make domain I more compact and stable on the one hand, but on the other hand the bulky side chains on the porphyrin ring would tend to prevent the heme-binding pocket of domain I from closing. The two opposing influences would then result in no net change in the stability or ellipticity of hemopexin at 233 nm.

One exception to this correlation in stability and ellipticity is SnPP. Freshly prepared SnPP–hemopexin complexes have a thermostability close to that of apohemopexin and an ellipticity close to that of heme–hemopexin. However, if the SnPP–hemopexin complex is left overnight at 4 °C, the ellipticity decreases to a value that is approximately the same as apohemopexin (data not shown). This does not occur with any of the other complexes. It may be that SnPP–hemopexin complexes have more than one conformational state, which would also help to explain the broad transition of SnPP–hemopexin in DSC (Figure 7c).

The heme-induced conformational changes in hemopexin are important for receptor-mediated heme transport, *e.g.*, heme–hemopexin has a higher affinity than apohemopexin for the mouse Hepa cell hemopexin receptor (Smith *et al.*, 1988). However, the exact nature of these conformational changes and the role of these changes in recognition of heme-hemopexin by its receptor remain unknown. The present results provide additional structure-based explanations and predictions for the binding of hemopexin to its receptor. For example, heme-hemopexin and CoPP-hemopexin (Smith *et al.*, 1993) are more compact (Morgan *et al.*, 1988b) and more stable (this work) and also have higher affinity for the hemopexin receptor (Morgan, 1976; Smith & Morgan, 1979) than apohemopexin. FeTPPS–hemopexin has essentially the same conformation and stability as apohemopexin (this work) and has an affinity for the hemopexin receptor comparable to that of apohemopexin (Smith *et al.*, 1988). From the present results, the affinity of a metalloporphyrin–hemopexin complex for the hemopexin receptor is predicted to be determined in large part by the stability of the complex, which can be approached experimentally by the thermostability of the complex. The more stable the metalloporphyrin–hemopexin complex, the higher the affinity of the complex for the receptor is expected to be. SnPP–hemopexin appears to be an exception in that it has an affinity to the hemopexin receptor like that of heme-hemopexin (Morgan *et al.*, 1988a) but a thermostability like that of apohemopexin. This is again probably due to the multiple states of the SnPP–hemopexin complex. Studies of the interaction of NiPP–hemopexin with the hemopexin receptor, which is predicted to have a lower affinity than apohemopexin, are in progress.

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