Genetics. In the article "Down syndrome phenotypes: The consequences of chromosomal imbalance" by J. R. Korenberg, X.-N. Chen, R. Schipper, Z. Sun, R. Gonsky, S. Gerwehr, N. Carpenter, C. Daumer, P. Dignan, C. Distėche, J. M. Graham, Jr., L. Hudgins, B. McGillivray, K. Miyazaki, N. Ogasawara, J. P. Park, P. Pagon, S. Pueschel, G. Sack, B. Say, S. Schuffenhauer, S. Soukup, and T. Yamanaka, which appeared in number 11, May 24, 1994, of Proc. Natl. Acad. Sci. USA (91, 4997–5001), the authors request that the following correction be noted. The name of an author, L. Hudgins, was misspelled. The title and authors should read as follows:

Down syndrome phenotypes: The consequences of chromosomal imbalance


Biochemistry. In the article “An arcane role of DNA in transcription activation” by Sangreyol Ryu, Susan Garges, and Sankar Adhya, which appeared in number 18, August 30, 1994, of Proc. Natl. Acad. Sci. USA (91, 8582–8586), a printer’s error resulted in the omission of the title and edited column heads from Table 1. The corrected table is shown here.

Table 1. Effect of increased distance and single-strand interruptions in the spacer on lac transcription

<table>
<thead>
<tr>
<th>Insertion interruption</th>
<th>Single-strand sequence</th>
<th>Spacer sequence</th>
<th>Cooperative RNAP binding</th>
<th>% lac transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. — —</td>
<td>CATTAGTTACCGAAGGGATCC</td>
<td>+</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2. — Nick</td>
<td>CATTAGTTAAGAGGGATCC</td>
<td>+</td>
<td>78.5 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>3. — 1-base</td>
<td>CATTAGCTACCGAAGGGATCC</td>
<td>+</td>
<td>53.6 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>4. — 2-base</td>
<td>CATTAGCTACCGAAGGGATCC</td>
<td>+</td>
<td>17.0 ± 17.4</td>
<td></td>
</tr>
<tr>
<td>5. — 4-base</td>
<td>CATTAGCTACCGAAGGGATCC</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6. 10 bp —</td>
<td>CATTAGTTAAGAGGGATCC</td>
<td>NT</td>
<td>51.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>7. 10 bp 6-base</td>
<td>CATTAGTTAAGAGGGATCC</td>
<td>NT</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

DNA templates carried the shown variations in the spacer region. The amount of transcription of each template interrupted in the bottom strand was normalized as percentage of the transcription obtained for the corresponding isogenic intact double-stranded linear template. The last column shows the average of several experiments, including the standard deviations, obtained from independent preparations of DNA. Results shown in line were obtained with DNA templates which were mixtures with nicks or gaps at the indicated position(s) of one or the other strand. Results were the same as shown in the last column when homogeneous single-stranded DNA circles with interruption in the bottom (as shown in column 4) or the top strand made by a bacteriophage λ replication-packaging system were used as templates. +, RNAP binding to wild-type DNA templates in the presence of CRP; NT, not tested.

Biochemistry. In the article “Roles of heme iron-coordinating histidine residues of human hemopexin expressed in baculovirus-infected insect cells” by Tomoko Satoh, Hiroyuki Satoh, Shin-ichiro Iwashara, Zbynek Hrkal, David H. Peyton, and Ursula Muller-Eberhard, which appeared in number 18, August 30, 1994, of Proc. Natl. Acad. Sci. USA (91, 8423–8427), the arrow was missing from Fig. 3 due to a printer’s error. The corrected figure and legend are shown here.
Roles of heme iron-coordinating histidine residues of human hemopexin expressed in baculovirus-infected insect cells

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ABSTRACT Hemopexin (Hx), the major heme-binding plasma glycoprotein, scavenges circulating heme and performs an antioxidant function. In the present study, human Hx was expressed in a baculovirus system and its presumed essential His residues were mutated to Thr as means of investigating their participation in heme binding. The recombinant Hx proteins were purified by sequential chromatography on Con A-agarose and SP-Sepharose. The purified recombinant wild-type Hx 2 and its heme binding. The binding constant for heme was considerably reduced, however, suggesting that glycosylation contributes critically to the heme binding property of Hx. Mutation either at His-127 or at His-56 plus His-127, but not at His-56 per se, reduced the affinity for heme by an order of magnitude relative to wild-type Hx. It is concluded that His-127 contributes to the high affinity for heme. We recorded proton NMR spectra to investigate the possibility that the degree of high-spin content is increased by deletion of an axial His–iron coordination. 1H NMR data indicate that each of the single-mutant heme–Hx complexes is predominantly low-spin, perhaps owing to coordination of the heme iron by the Thr side-chain oxygen or water oxygen coordinating to the iron.

Heme in tissues and circulation can be toxic when interacting with oxygen by way of radical formation (see review in ref. 1; ref. 2). The abundant plasma β1-glycoprotein hemopexin (Hx) binds heme in an equimolar ratio with an exceptionally high affinity. The dissociation constant (Kd) of heme–Hx is <10^{-11} M (3), the highest affinity of any known characterized heme-binding protein (4). Hx serves two functions as a heme transporter: scavenging of circulating heme for iron conservation (5, 6) and prevention of oxidative tissue damage caused by heme-catalyzed radical formation (7, 8).

Human plasma Hx consists of a single polypeptide chain containing 439 amino acid residues and has a molecular mass of 60 kDa, 20% of which is carbohydrate (9). Human Hx is cleaved after limited digestion by trypsin into two homologous domains, of which the N-terminal domain binds heme (10), as is likewise found for rabbit Hx (11).

The identity of the amino acids coordinating heme binding of Hx has not been firmly established. It has been suggested that two His side chains act as heme axial ligands of Hx, according to results obtained by electron paramagnetic resonance (12, 13), magnetic circular dichroism (14), 1H NMR studies (15) and chemical (16) and photochemical (17) modifications. Even protoporphyrin IX is bound by Hx with a Kd of 10^{-10} M (18, 19), although this porphyrin lacks the central iron. Two histidines, presumably acting as axial ligands, may be essential for the exceptionally high affinity that Hx has for heme. The protein whose His residues were modified (16, 17) retained a reduced ability to bind heme.

There are five conserved His residues in the N-terminal domain of human and rat Hx. Two of them, His-56 and His-127, are located in a particularly highly conserved region and are, therefore, the most likely candidates for heme axial ligands (20). His-56 and His-127 of rabbit Hx were also suggested as heme iron-coordinating residues according to homology analysis, chemical modification, and antibody binding data (21).

A combination of site-directed mutagenesis and functional (heme binding assay) analyses should be particularly useful to investigate whether these His residues are the heme iron axial ligands of human Hx and whether they contribute energetically to the stability of the complex. Because Hx is an extensively glycosylated protein, only eukaryotic systems can be used that provide sufficient posttranslational modifications to afford heme binding. Initial attempts to express human Hx in an Escherichia coli system produced a nonglycosylated protein that was unable to bind heme (T.S., H.S., and U.M.-E., unpublished data).

In the present work, we expressed recombinant human Hx in a baculovirus system and confirmed, by site-directed mutagenesis analyses, that His-127 contributes to the high affinity of Hx for heme. In addition, mutations at His-56 plus His-127 caused changes in the optical absorbance and the 1H NMR spectrum of the complex. Surprisingly, the His-56 mutation per se caused no loss of heme binding in the recombinant proteins.

MATERIALS AND METHODS

Construction of Recombinant Baculovirus Containing Human Hx cDNA. All restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs. A 7-deaza sequencing kit and Sequenase version 2.0 were from United States Biochemical. All basic recombinant techniques were done according to standard protocols (22). Construction of the recombinant human Hx linked to the leader sequence of honeybee melittin (23) were as follows. cDNA encoding a human Hx (S. Metcalfe and U.M.-E., unpublished data) was double-digested by Spe I and Pst I and cloned into a pSL301 vector (Invitrogen). A synthetic double-stranded oligodeoxynucleotide linker corresponding to the melittin leader sequence was cloned into the Hx construct between HindIII and Spe I sites. The nucleotide and amino acid sequences of the linker were as follows.

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Abbreviation: Hx, hemopexin.

†To whom reprint requests should be addressed.
The melittin (leader sequence)–Hx fusion cDNA was excised by Nhe I digestion and subcloned into a pBacPAK8 transfer vector (Clontech) at the Xba I site (Nhe I and Xba I restriction ends are compatible). The correctness of the constructs was verified by restriction mapping and sequencing. The melittin–Hx construct in the pBacPAK8 vector was then cotransfected into Spodoptera frugiperda Sf9 cells with linearized BacPAK6 viral DNA (Clontech) by means of cationic liposomes (Lipofectin, GIBCO/BRL). Plaques of the recombinant baculovirus expressing human Hx were selected by standard methods (24).

Site-Directed Mutagenesis. The human Hx cDNA joined to the melittin leader sequence in the pSL301 vector was subcloned into a Bluescript II KS(+) phagemid vector (Stratagene) between the HindIII and BamHI sites. Single-stranded plasmid DNAs were rescued by infecting Ra08 helper phage. Site-directed mutagenesis was performed with a kit from Promega. Mutant primers were the complementary strands of the sequences summarized in Fig. 1. His-56, -82, and -127 of human Hx were mutated to Thr (H56T, H82T, and H127T mutants), because these mutations enabled us to introduce new restriction sites on the Hx cDNA. The mutants were then cleaved by Nhe I and subcloned into a pBacPAK8 baculovirus transfer vector at the Xba I site, as described above.

Cell Cultures. For propagation of recombinant baculoviruses carrying melittin–Hx fusion cDNA, SF21 cells were infected and cultured at 27°C in TNM-FH medium (23). For expression of recombinant Hx, High Five insect cells (Invitrogen) were cultured in serum-free Ex-cell 400 medium (JRH Biosciences, Lenexa, KS) supplemented with gentamicin (50 μg/ml) and amphotericin B (2.5 μg/ml).

Expression and Purification of Recombinant Wild-Type and Mutant Hxs. High Five cells were infected with the human Hx cDNA-containing virus at a multiplicity of 10. Forty-eight hours later, conditioned medium was collected and dialyzed overnight at 4°C against 0.5 M NaCl/20 mM Tris Cl, pH 7.4 [equilibration buffer for Con A-agarose (Sigma)] at 4°C overnight. The sample was then loaded onto a Con A-agarose column and bound proteins were eluted with 0.3 M methyl α-D-mannoside in equilibration buffer. The final step was chromatography on SP-Sepharose fast flow (Pharmacia), using a linear gradient of 10–100 mM sodium citrate, pH 5.9. Fractions containing the recombinant Hx were identified by Western blot analysis using anti-human Hx polyclonal antibody.

Heme Titration. Concentrations of recombinant Hx in 50 mM sodium phosphate buffer (pH 7.4), were determined spectrophotometrically using extinction coefficients of 118 mM−1cm−1 at 280 nm for apo-Hx and of 131 mM−1cm−1 at 280 nm for heme–Hx (25). Concentrations of heme, dissolved in 40% dimethyl sulfoxide, were determined spectrophotometrically by using an extinction coefficient (mM−1cm−1) of 180 at 400 nm (26).

Heme binding to the recombinant Hx was monitored by absorption spectroscopy in the Soret region and by fluorescence quenching measurements of Hx tryptophan residues. To 1 ml of 1 μM Hx, sequential 20-μl aliquots of 13.1 μM heme were added. After a 1:1 molar ratio of heme to Hx was reached, the heme–protein complexes were chromatographed on a DE-52 DEAE-cellulose (Whatman) column and eluted with phosphate-buffered saline to remove loosely bound heme. The absorption spectra were recorded at 240–500 nm on a Hitachi U-2000 absorption UV/visible spectrophotometer.

The Kd values were measured by fluorescence quenching in a 1-cm-path-length cell at room temperature at a protein concentration of 1 μM with a Perkin–Elmer MPF-44 fluorescence spectrophotometer. The excitation and emission wavelengths were 280 nm and 300–340 nm, respectively. The relative quenching of the fluorescence emission at 333.5 nm was plotted against the heme concentration. The Kd values were determined by computer fitting the data by the binding isotherm method (27).

RESULTS AND DISCUSSION

Expression of Recombinant Hx in a Baculovirus System. High Five insect cells (Invitrogen) were infected with recombinant baculovirus containing human Hx cDNA. Secretion of recombinant Hx was monitored by SDS/PAGE and Western blot analysis at 0, 24, 48, 72, 96, and 120 hr after infection (Fig. 2). Substantial amounts of recombinant Hx were produced at 24 hr, and maximal secretion was reached between 48 and 72 hr. Similar time courses of secretion have been reported for other proteins—e.g., human plasma cholesteryl ester transfer protein (28), human factor VIII (29), and bovine cytochrome P-450 (30).

Purification and Characterization of Recombinant Human Hx. Both human plasma Hx and recombinant human Hx were

![Fig. 1. Mutant sequences.](image-url)

![Fig. 2. Time course of recombinant wild-type Hx secretion from High Five insect cells. At 24 hr intervals postinfection, conditioned media of High Five cells (5 × 10² cells per sample) infected with recombinant virus containing human Hx cDNA were analyzed by SDS/10% PAGE (A) and Western blot with anti-human Hx polyclonal antibody (B). Numbers above the application slots indicate time (hours) postinfection. Recombinant Hx was present at 24 hr and expressed maximally between 48 and 72 hr postinfection. The molecular mass of the recombinant Hx is estimated to be 55 kDa, whereas that of plasma Hx (1 μg) is 60 kDa.](image-url)
positive on lectin staining using the Con A-peroxidase method (31, 32) on Western blots. For this reason, we used Con A-agarose chromatography as the first purification step to separate glycosylated proteins, followed by SP Sepharose chromatography. After Con A-agarose chromatography the recombinant Hx showed one major band (Fig. 3, lane 3). No obvious contaminant was detected by SDS/10% PAGE after SP Sepharose chromatography (lane 4).

The apparent molecular mass of the recombinant Hx was 55 kDa, whereas that of plasma Hx is 60 kDa (4). The decreased molecular mass of the recombinant protein is probably due to the difference in glycosylation between insect and mammalian systems. Deglycosylation of both plasma Hx and recombinant Hx by N-glycosidase F (New England Biolabs) showed an identical molecular mass on SDS/PAGE, ≥49 kDa (data not shown), as predicted (33). The carbohydrate content of the recombinant Hx was, therefore, about 10% of the mass.

The N-terminal amino acid sequence of the purified recombinant Hx was determined in order to ascertain that the melittin leader peptide had been removed. The N-terminal Thr residue of the human plasma Hx is blocked because of O-linked glycosylation (34). As expected, the N-terminal amino acid residue of the recombinant Hx was unidentifiable; however, the amino acid sequence from 2 to 12 matched that of plasma Hx (data not shown). This result indicated that the melittin leader peptide had been cleaved correctly and that the N-terminal amino acid residue of recombinant Hx had been modified, presumably by glycosylation.

**Functional Analyses of the Recombinant Human Hx Reveals Retention of Heme Binding Activity.** The heme binding ability of the recombinant Hx was compared with that of plasma Hx by absorption spectroscopy (Fig. 4). The absorption spectrum of the recombinant heme–Hx exhibited a peak at 413.5 nm, characteristic of heme–Hx purified from plasma (35). The absorption peak at 413.5 nm of the recombinant Hx was only slightly lower than that of the plasma Hx. The slightly lower absorption of the recombinant Hx might be due to the difference in glycosylation between recombinant and native proteins, which could affect the tertiary structure of the protein. Recombinant Hx that we expressed in an E. coli system did not retain heme binding ability and was rapidly degraded (T.H., S.H., and U.M.-E., unpublished data). Glycosylation may contribute to the high binding affinity of Hx for heme. An analysis of the affinity of the recombinant Hx for heme is discussed below.

**Expression and Characterization of Site-Directed Hx Mutants.** His-56 and His-127 have been predicted to be the heme iron-coordinated amino acid residues of rat and human Hx

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**FIG. 3.** SDS/PAGE of recombinant wild-type Hx after each purification step. Aliquots (5 μg) were analyzed by SDS/4–20% gradient PAGE and stained with Coomassie brilliant blue R-250. Lanes: 1, molecular size markers; 2, conditioned medium containing recombinant Hx after infection; 3, following Con A-agarose chromatography; 4, eluate from SP-Sepharose chromatography. Arrow indicates the recombinant Hx at 55 kDa.

**FIG. 4.** Absorption spectra of plasma and recombinant wild-type Hx complexes with heme. Heme was mixed with plasma Hx or recombinant Hx in an equimolar ratio. The spectra of heme–plasma Hx (spectrum A) and heme–recombinant Hx (spectrum B) were recorded from 240 to 500 nm. The heme–recombinant Hx complex shows an absorption peak at 413.5 nm, as is observed for the heme–plasma Hx, indicating that the recombinant Hx binds heme.

**FIG. 5.** SDS/PAGE and Western blot analyses of the recombinant wild-type Hx and His→Thr mutants. The His residues of the recombinant Hx at 56, 82, and 127 were mutated to Thr. Each conditioned medium containing recombinant Hx or its mutants (5 μg) was analyzed by SDS/10% PAGE with Coomassie brilliant blue R-250 staining (A) and Western blot probed with anti-human Hx polyclonal antibody (B). Lanes: P, plasma Hx; 1, wild-type Hx; 2, H56T; 3, H82T; 4, H127T; 5, H56T/H127T.
nearly identical to that of the wild-type Hx. The $K_d$ values of wild-type and mutant Hx proteins determined by fluorescence quenching (Fig. 7) were $3 \times 10^{-8}$ M, $5 \times 10^{-8}$ M, $4 \times 10^{-7}$ M, $2 \times 10^{-7}$ M, and $4 \times 10^{-7}$ M for the wild-type and the H56T, H82T, H127T, and H56T/H127T mutants, respectively. That the wild-type and mutant proteins display a lower affinity for heme than does plasma Hx [$K_d < 10^{-13}$ M (3)] may be the result of tertiary structure differences caused by incomplete glycosylation or differences in glycosylation, or even lack of pivotal participation by specific glycosyl groups in the heme binding site. On the contrary, the further decrease in $K_d$ for heme binding of the mutants including His-127 may be due to the unique His-iron coordination.

$^1$H NMR spectra are very sensitive to the axial ligation state in low-spin heme proteins (37). We have already reported that the pattern of hyperfine-shifted resonances of heme–Hx is species dependent (15). Fig. 8 shows traces for the downfield hyperfine-shifted portion of the spectra. The recombinant material (Fig. 8, spectrum B) was very similar to the native material (spectrum A; ref. 15). Minor differences can be expected, arising from variations in temperatures used for measurements as well as the extent of glycosylation of the protein. Replacement of His-82, while not changing the binding affinity of Hx for heme, did cause minor changes in the $^1$H NMR spectrum C relative to spectrum B. Surprisingly, replacement of His-56 or His-127 (spectra D and E) resulted in a nearly identical spectrum. Loss of either His-iron coordination normally leads to a high-spin complex.

![Absorbance vs Wavelength (nm)](image)

**Fig. 6.** Absorption spectra of recombinant wild-type Hx and His→Thr mutants complexed with heme. Absorption spectra of heme–Hx complexes, after passage through a DE52 column, were monitored from 240 to 500 nm. Recombinant wild-type Hx and H56T and H82T mutants show identical absorption spectra (spectrum A). The absorption peak at 413.5 nm of a H127T is decreased significantly (spectrum B). H56T/H127T shows no absorption peak at 413.5 nm (spectrum C).

![Bound heme vs Free heme](image)

**Fig. 7.** Heme binding to recombinant wild-type Hx and His→Thr mutants. Curves for binding of heme to Hx were obtained from fluorescence quenching data. The fluorescence quenching was measured at a protein concentration of 1 μM and the relative quenching of fluorescence emission at 353.5 nm was determined by addition of heme to Hx solutions. ○, Recombinant Hx ($K_d = 3 \times 10^{-8}$ M); □, H56T ($K_d = 5 \times 10^{-8}$ M); ◇, H82T ($K_d = 4 \times 10^{-8}$ M); ▲, H127T ($K_d = 2 \times 10^{-7}$ M); ●, H56T/H127T ($K_d = 4 \times 10^{-7}$ M).

![Chemical shift vs Temperature (°C)](image)

**Fig. 8.** $^1$H NMR spectra of the downfield resolved region of the recombinant heme–Hx complexes. Spectra: A, heme–Hx isolated from human plasma (170 μM); B, heme–wild-type Hx (90 μM); C, heme–H82T Hx (30 μM); D, heme–H56T Hx (90 μM); E, heme–H127T Hx (50 μM); F, heme H56T/H127T Hx (30 μM). Spectrum A was recorded at 40°C and spectra B–F at 25°C, all at pH 7.0. Spectra B–E are very similar, indicating a large low-spin iron content and similar magnetic axis orientation.

![Absorption vs Wavelength (nm)](image)

nearly identical chemical shifts of the resonances shown in Fig. 8, spectra A–E, indicate that the heme–iron complex is still largely low-spin (indicative of a six-coordinate heme iron). Many of these resonances arise from the heme methyls, as demonstrated with deuterated heme using the rabbit protein (15). It is unfortunate that the exceedingly limited amount of material did not allow measurement of either T1 relaxation or linewidths (T2), which would have diagnostic of the spin state. The linewidths in the His-127 mutant appeared to be larger, suggesting the presence of an increased high-spin content (indicative of a five-coordinate heme iron). Under the assumption that both His-59 and His-127 coordinate the iron in heme–Hx purified from plasma, it may be that the Thr-for-His mutation allows for coordination of the Thr side-chain oxygen to the iron. It is also possible that a water oxygen is coordinated to the iron. The remaining His–iron bond may be so strong that interaction with an oxygen at the alternative axial coordination site is sufficient to give substantial low-spin character to the complex. This may be why heme binding is reduced so little in these mutants. Although a low-spin (predominantly $S = 1/2$) complex with oxygen–iron coordination would be unusual, it has precedent when there is an appropriate electrostatic field provided by the protein matrix (38).
Fig. 9. Visible-region optical spectra of the recombinant heme–Hx complexes: A, heme–wild-type Hx; B, heme–H82T Hx; C, heme–H56T Hx; D, heme–H127T Hx; E, heme–H56T/H127T Hx. The spectra were recorded at room temperature and pH 7 with heme/Hx ratios < 1.0 to ensure that the spectrum was that of the complex. The Soret maximum is shifted to a shorter wavelength by 1 nm in spectra D and E, both of which represent mutants with His-127 changed to Thr, arb, Arbitrary units.

At present we have no explanation for why the chemical shift pattern remains so nearly constant in Fig. 8, spectra B–E. Only substitution of both His-56 and His-127 caused loss of the hyperfine-shifted resolved resonances between 32 and 12 ppm (Fig. 8, spectrum F). Yet it is evident that heme is still bound to mutant His-127, even metal-free protoporphyrin IX is bound by Hx (18, 19). The optical spectrum (Fig. 6, spectrum B) and the $k_{u}$ values of the H127T mutant confirm that the heme is bound (Fig. 9, spectrum D). At heme/Hx ratios < 1, the Soret maximum is consistently at 413.5 nm, except for the mutants involving His-127, for which the Soret peak is shifted to higher energy by $\approx 1$ nm (Fig. 9).

These results indicate that His-127 may play a more pivotal role for binding of heme by Hx than does His-56. Alternatively, cyanide ion binds to the central iron of heme–Hx in a pseudo-first-order reaction, suggesting that cyanide ion competes with one of the iron–His interactions (39). His-56 of human Hx may be the target of the cyanide ion. The tentative conclusion is drawn that mutation at His-56 causes no significant loss of heme binding activity in comparison to that of the wild type, whereas mutation of His-56 plus His-127 reduces the binding constant by a factor of $\approx 10$. These data suggest that His-56 plays merely a supportive function. It appears that glycosylation may also play a role in heme binding.

The physiological functions of Hx are (i) scavenging of circulating free heme to prevent radical formation and (ii) heme transport to the liver for iron reutilization. As a heme scavenger, Hx needs to bind free heme rapidly and tightly, while as a transporter, Hx needs to release heme efficiently. It is likely that His-56 and appropriate glycosylation are important contributors for proper formation of the heme pocket of Hx. When Hx needs to release heme, conformational changes may occur that open the heme pocket of Hx. The lower affinity of His-56 for heme in combination with the higher affinity of His-127 might enable Hx, alternatively, to bind heme tightly, and to release it efficiently once the heme His-127 bond is destabilized.

We thank Dr. Monica Laskey for her kind advice in the early part of this project. This work was supported by grants from the National Institutes of Health (DK30203 and DK30664) and the Robert Leet and Clara Guthrie Patterson Trust and by the Medical Research Foundation of Oregon.