Hemin Interactions and Alterations of the Subcellular Localization of Prion Protein

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Hemin (iron protoporphyrin IX) is a crucial component of many physiological processes acting either as a prosthetic group or as an intracellular messenger. Some unnatural, synthetic porphyrins have potent anti-scrapie activity and can interact with normal prion protein (PrP\textsuperscript{C}). These observations raised the possibility that hemin, as a natural porphyrin, is a physiological ligand for PrP\textsuperscript{C}. Accordingly, we evaluated PrP\textsuperscript{C} interactions with hemin. When hemin (3–10 μM) was added to the medium of cultured cells, clusters of PrP\textsuperscript{C} formed on the cell surface, and the detergent solubility of PrP\textsuperscript{C} decreased. The addition of hemin also induced PrP\textsuperscript{Sc} internalization and turnover. The ability of hemin to bind directly to PrP\textsuperscript{C} was demonstrated by hemin-agarose affinity chromatography and UV-visible spectroscopy. Multiple hemin molecules bound primarily to the N-terminal region of PrP\textsuperscript{C} with reduced binding to PrP\textsuperscript{Sc} containing residues 34–94. These hemin-PrP\textsuperscript{C} interactions suggest that PrP\textsuperscript{C} may participate in hemin homeostasis, sensing, and/or uptake and that hemin might affect PrP\textsuperscript{C} functions.

Iron protoporphyrin IX, a natural cyclic tetrapyrrole (cTP),\textsuperscript{2} is vital to cellular homeostasis in either the Fe\textsuperscript{3+} (hemin) or Fe\textsuperscript{2+} (heme) oxidation state (supplemental Fig. 1). In hemoglobin and myoglobin the reversible binding of oxygen to the reduced iron of the heme permits oxygen transport and storage (1). Heme also plays key roles in the electron transport function of various cytochromes and in the catalytic reactions of hydrogen peroxide by catalases and peroxidases (1, 2). Hemin may inhibit PrP\textsuperscript{Sc} formation. These findings suggest that PrP\textsuperscript{C} may be involved in copper transport or homeostasis (18, 19). Interestingly, molecules such as glycosaminoglycans and other anti-transmissible spongiform encephalopathy compounds also bind to the N terminus and alter the subcellular trafficking of PrP\textsuperscript{C} (20–23). This suggests that the modulation of endocytosis of PrP\textsuperscript{C} through its N-terminal domain is important in a conserved physiological function of PrP\textsuperscript{C}.

Based on these observations, we evaluated the possibility of hemin being a physiological ligand of PrP\textsuperscript{C}. Here we show that hemin promotes PrP\textsuperscript{Sc} clustering, internalization, and degradation in cultured cells. In cell-free reactions, the binding of PrP\textsuperscript{C} to hemin alters the aggregation state and inherent peroxidase activity of the latter.

**EXPERIMENTAL PROCEDURES**

Preparation of Recombinant PrP (rPrP\textsuperscript{C})—Cell pellets of *Escherichia coli* expressing hamster PrP corresponding to residues 23–231 or 90–231 in the pET41 vector (EMD Biosciences) were lysed with BugBuster\textsuperscript{TM} and lysonase (EMD Biosciences) in the presence of EDTA-free protease inhibitors (Roche Applied Science). Inclusion bodies were washed twice with 0.1× BugBuster\textsuperscript{TM} in water and pelleted by centrifugation. The enriched rPrP\textsuperscript{C} was further purified by minor modifications to the method of Zahn et al. (24). The protein was eluted with 10 mM sodium phosphate (pH 5.8), 500 mM imidazole, and 10 mM Tris. Pooled fractions were dialyzed against 10 mM sodium acetate or PBS. The construct containing residues 23–106 was...
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purified in the same manner except the protein was eluted from the nickel column using 10 mM sodium acetate at pH 3.5, and fractions that contain the protein were further purified on an SP-Sepharose column using a salt gradient in sodium acetate at pH 5.0. The protein concentration of rPrPC was determined by absorbance at 280 nm. Purity of the final protein preparations was estimated at ≥99% when analyzed by SDS-PAGE, Western blot, and matrix-assisted laser desorption ionization-mass spectrometry (data not shown).

Preparation of Solutions—Hemin (Mann Research Laboratories Inc.), biliverdin (Frontier Scientific), and bilirubin (Frontier Scientific) were dissolved in Me₂SO at 10 mM. Further dilutions were carried out in PBS or serum-free Opti-MEM (Invitrogen). Hemin stock solutions were also prepared in 0.5 M NaOH at 10 mM to investigate the effect of the μ-oxo-dimer of hemin, which is known to form at basic pH.

UV-visible Absorption Spectroscopy—For spectroscopic analysis, rPrPC and hemin were mixed in PBS (pH 7.4) containing 1 mM EDTA prior to measurement of absorbance. Measurements were made on a SpectraMAX 190 plate reader (Molecular Devices). The spectra were acquired between 300 and 800 nm.

Cell Culture—N2a5E4E is a mouse neuroblastoma (N2a) cell line that overexpresses mouse PrPC as described previously (25). N2aGFP-GPI is an N2a cell line that was stably transfected with a GFP-GPI (glycosylphosphatidylinositol-anchored green fluorescent protein) expression vector (26). CF10 cells generated from PrPC null mice (27) were transduced with a murine retroviral vector (pSFF) encoding full-length hamster PrPC or hamster PrPC lacking residues 34–94 (28). Human neuroblastoma cells (NB1) express endogenous levels of PrPC. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in Opti-MEM supplemented with 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin (100 units/ml, 100 μg/ml; Invitrogen).

PrPC Binding to Hemin-Agarose—A confluent tissue culture flask (25 cm²) containing cells described above was rinsed three times with PBS and lysed with 600 μl of PBS containing 0.5% Triton X-100 and 0.5% sodium deoxycholate. Cell debris and nuclei were removed by centrifugation at 2,700 × g for 5 min, and 600 μl of postnuclear supernatant was recovered. To the postnuclear supernatant, a protease inhibitor mixture (Complete, Roche Applied Science) was added according to the manufacturer’s instructions. The final concentration of NaCl was adjusted to 0.5 M. Additional Triton X-100 and sodium deoxycholate were added to final concentrations of 1%. Sarkosyl (1%) was also added to improve the dissolution of the cell membranes. The final volume was adjusted to 1200 μl after the addition of all reagents and then incubated for 10 min at room temperature to allow the dissolution of membranes. Hemin-agarose beads (Sigma) were washed three times with PBS prior to use. Washed beads containing the equivalent of 0.1 μmol of hemin were added to 200 μl of cell lysate and incubated for 10 min at room temperature. After incubation, the unbound fraction was collected and precipitated with 800 μl of methanol. The beads were washed three times with the same buffer used for the binding step. The methanol precipitate and the beads were resuspended in 100 μl of SDS-PAGE sample buffer and boiled, and 5 μl was subjected to SDS-PAGE with staining for proteins with GelCode Blue (Pierce) or to immunoblot analysis for PrPC using antibody D13 (InPro) for mouse and hamster PrPC and 3F4 for human PrPC.

Immunodetection of Cell Surface PrPC—N2a5E4E cells were plated at low density in a 96-well plate and grown to confluence. At confluence, cells were washed once with serum-free Opti-MEM and treated with hemin, biliverdin, or bilirubin at 0, 1, 3, and 10 μM for 1 h at 37 °C. After treatment, the cells were fixed with 4% paraformaldehyde in PBS for 10 min followed by two washes with PBS. Then the cells were incubated with antibody D13 (InPro) diluted in PBS at 1:1000 for 1 h. After three washes of 5 min each, cells were incubated with secondary antibody conjugated with alkaline phosphatase diluted in PBS at 1:2000 for 1 h. Cells were washed three times and incubated with AttoPhos substrate (Promega) for 7–15 min until a yellow color was visible. Fluorescence intensity was measured in a SpectraMAX Gemini EM plate reader (Molecular Devices) using 450 nm excitation filter and 520 nm emission filter. The relative fluorescence intensity was calculated based on the signal obtained from untreated cells.

Immunofluorescence—N2a5E4E cells were washed twice with serum-free Opti-MEM and treated with 3 μM hemin for 1 h. After treatment, cells were fixed with 4% paraformaldehyde in PBS for 10 min followed by two washes with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. To block nonspecific antibody binding, cells were incubated with 10% normal goat serum and 0.1% Triton X-100 in PBS (blocking solution) for 10 min. An antibody against PrPC, SAF-32 (Cayman Chemicals), was diluted in blocking solution (1:200) and added to the cells. After 1 h of incubation, the cells were washed three times with PBS and incubated with secondary antibody anti-mouse IgG conjugated with Alexa 488 fluorescent dye (1:1000) for 1 h. Cells were washed three times and observed by confocal microscopy. All images were acquired with the same confocal parameters.

Biocytin and Isolation of Cell Surface Proteins—N2aGFP-GPI cells were plated in 24-well plates and cultured for 3 days. At confluence, cells were washed three times with serum-free Opti-MEM and treated with hemin at 0, 1, 3, and 10 μM for 1 h at 37 °C. The cells were washed three times with PBS containing 1 mM CaCl₂ and 1.2 mM MgSO₄ (PBS Ca²⁺/Mg²⁺) on ice. Then 150 μl of 1 mg/ml NHS sulfo-LC biotin was added per well and incubated for 5 min at room temperature. NHS sulfo-LC biotin reacts predominantly with primary amino groups. After biotinylation, the cells were washed three times with PBS Ca²⁺/Mg²⁺ containing 100 mM glycine. The cells were then lysed with 200 μl of PBS containing 0.5% Triton X-100, 0.5% sodium deoxycholate, and a protease inhibitor mixture (Complete, Roche Applied Science) (lysis buffer). Cell lysates were incubated with 20 μl of streptavidin Dynabeads (Invitrogen) for 30 min at room temperature and then washed three times with the lysis buffer. The beads were resuspended in 40 μl of SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE and Western blot analysis using the designated antibody for PrPC (D13, InPro), GFP (monoclonal anti-GFP, Roche Applied Science), or NCAM (anti-NCAM, Chemicon). Biotinylated proteins in general were stained with Neutradvin.
conjugated with alkaline phosphatase (Pierce). To check the effect of hemin on PrP\(^\text{Sc}\) turnover, biotinylation of N2a5E4E cells was performed as described above but prior to the hemin treatment.

**Detergent Insolubility Assay**—All cells described were cultured and treated with hemin as described in the section above. The cells were then lysed with 200 µl of lysis buffer. Nuclei and cell debris were removed by centrifugation at 2,700 \(\times g\) for 5 min at 4°C, and then Sarkosyl was added to a final concentration of 0.5–1% (29). After 10 min of incubation on ice or at 37°C, the detergent-insoluble material was recovered by ultracentrifugation at 360,000 \(\times g\) for 30 min at 4°C. Supernatant proteins were subjected to methanol precipitation. Pellets that were generated from ultracentrifugation or methanol precipitation were dissolved in SDS-PAGE sample buffer and subjected to immunoblot analyses using antibodies described in the section above.

**Metabolic Labeling**—Tissue culture flasks (25-cm\(^2\)) were seeded with equal numbers of human neuroblastoma NB1 cells and grown until they were 80–90% confluent. The cells were preincubated for 1 h with 5 ml of 10 \(\mu\)M hemin in serum-free Opti-MEM followed by a 30-min incubation in 2 ml of methionine-free MEM containing hemin. Then 500 \(\mu\)Ci of \([^{35}\text{S}]\)methionine was added to each flask and incubated for 30 min. Cells were rinsed twice with PBS and incubated in serum-free Opti-MEM containing hemin for the designated chase time.

**Peroxidase Activity**—The peroxidase activity was measured by oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) (Pierce) or 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-di-ammonium salt (Pierce) by \(\text{H}_2\text{O}_2\). Hemin (8 \(\mu\)M) was mixed with various concentrations of rPrP\(^\text{Sc}\), prior to the addition of substrate. After substrate addition the reaction was monitored for absorbance at 650 nm on a SpectraMAX 190 plate reader (Molecular Devices).

**RESULTS**

**Hemin-induced PrP\(^\text{Sc}\) Clustering and Internalization**—Several different inhibitors of PrP\(^\text{Sc}\) formation, e.g. pentosan polysulfate (21), copper (18), suramin (30), and phosphorothioate oligonucleotides (22), affect the intracellular localization of PrP\(^\text{C}\). Because various synthetic cTPs inhibit PrP\(^\text{Sc}\) formation, we wondered if hemin, as a natural cTP and potential physiological ligand for PrP\(^\text{C}\), can also affect PrP\(^\text{C}\) localization. This was tested initially by using an immunofluorescence assay for PrP\(^\text{C}\) detection in fixed and permeabilized cells. To enhance PrP\(^\text{C}\) detection, a neuroblastoma cell line that expresses a high level of PrP\(^\text{C}\), N2a5E4E, was used. Without hemin treatment, both cell surface and intracellular perinuclear staining was observed. Hemin (3 \(\mu\)M) treatment of N2a5E4E cells for 1 h decreased the immunofluorescence of PrP\(^\text{C}\) on the cell surface and caused some residual surface staining to appear more punctate than in the control cells (Fig. 1). No staining was observed when the primary antibody SAF-32 was omitted from the staining protocol. Furthermore, SAF-32 did not stain CF10 cells generated from PrP\(^\text{C}\) null mice (data not shown) confirming the specificity of the antibody against PrP\(^\text{C}\). Finally, similar results were obtained with antibody D13 (data not shown). These hemin effects on PrP\(^\text{C}\) localization were not likely because of cytotoxicity because the treatment at \(\leq 10 \mu\)M for at least 4 h did not induce any signs of toxicity as judged by morphology or a cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (data not shown). Altogether, these results suggested that hemin induced both the aggregation and internalization of PrP\(^\text{C}\).

**Selective Effects of Hemin on PrP\(^\text{C}\) Aggregation**—The formation of PrP\(^\text{Sc}\) aggregates was also evaluated using a detergent insolubility assay (29). Upon hemin treatment, PrP\(^\text{C}\) solubility decreased in a dose-dependent manner in both mouse and human cell lines (Fig. 2, a, b, e, and f). To eliminate the possibility of PrP\(^\text{Sc}\) aggregation being due to overexpression, the solubility of endogenous PrP\(^\text{C}\) expressed in N2aGFP-GPI cells was also analyzed, and similar results were observed (supplemental Fig. 2). Because hemin can interact with many proteins, we investigated the selectivity of the effects of hemin on the aggregation of PrP\(^\text{C}\) compared with other cell surface proteins such as NCAM (neural cell adhesion molecule), which interacts with PrP\(^\text{C}\) (31, 32), and GFP-GPI protein, which follows default trafficking pathways of GPI anchored, lipid-raft-associated proteins (26). No hemin-induced alteration of NCAM or GFP-GPI solubility was observed, indicating a degree of specificity for the effects of hemin on PrP\(^\text{C}\) solubility (Fig. 2c and supplemental Fig. 2).

**Quantifying Hemin-induced PrP\(^\text{C}\) Internalization**—To estimate the extent of internalization of PrP\(^\text{Sc}\) after hemin treatment, relative amounts of cell surface PrP\(^\text{C}\) were quantified using a fluorogenic immunoassay described under “Experimental Procedures.” To increase the PrP\(^\text{C}\) detection in this assay, N2a5E4E cells were used. The PrP\(^\text{C}\) specificity of the assay was indicated by the lack of fluorescence signal when the primary antibody (D13) was omitted and when the assay was applied to primary neuronal cells derived from PrP\(^\text{C}\) null mice. The treatment of N2a5E4E cells with hemin for 1 h caused a dose-dependent reduction of cell surface PrP\(^\text{C}\) (Fig. 3) reaching \(\sim 10\%\) of control levels at 10 \(\mu\)M hemin. It is known that basic solutions (e.g. NaOH) favor the formation of \(\mu\)-oxo-dimers of hemin. NaOH-treated hemin was slightly more potent than Me\(_2\)SO-solubilized hemin at causing PrP\(^\text{C}\) internalization at 3 \(\mu\)M, but both solutions were effective overall. In contrast to hemin, its linear tetrapyrrole metabolites biliverdin and bilirubin did not significantly reduce cell surface PrP\(^\text{C}\) (Fig. 3).
Selectivity of Hemin Effects on PrPC Internalization—To further test the selectivity of hemin effects on PrPC internalization, N2aGFP-GPI cells that express endogenous levels of PrPC and a recombinant GFP-GPI were treated with hemin, and cell surface proteins were then biotinylated, captured with streptavidin-coated magnetic beads, and subjected to SDS-PAGE. The gels were either immunoblotted for the detection of individual proteins or stained with a Neutravidin-alkaline phosphatase conjugate to reveal the overall profile of biotinylated cell surface proteins. Consistent with previous assays, the cell surface PrPC signal decreased in a dose-dependent manner (Fig. 4, a and b), but the extent of internalization was lower than the previous assay using the N2a5E4E cells. This discrepancy could be related to different expression levels of PrPC in the two cell types because the N2aGFP-GPI cells express a lower, endogenous level of PrPC, whereas the N2a5E4E cells overexpress PrPC. In contrast, the banding patterns and intensity of many other cell surface proteins were not visibly altered with hemin treatment (Fig. 4, a). We also examined hemin effects on NCAM and GFP-GPI. As shown in Fig. 4, a and b, no alteration of NCAM was observed, whereas GFP bands increased with hemin treatment. GFP-GPI, expressed under a cytomegalovirus promoter whose activity can be enhanced by histone acetylation or demethylation (33), given that hemin can regulate both histone acetylation and methylation (34), the increase of GFP-GPI on the cell surface could be due to increased expression, which was confirmed by a Western blot assay on total cell.
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In the immunofluorescence studies of PrP^C using N2a5E4E cells, we observed that in some cells, intracellular fluorescence increased as the cell surface staining decreased. However, in other cells, the overall fluorescence intensity decreased (Fig. 1) suggesting that the internalized PrP^C might have been degraded. To directly analyze whether degradation of PrP^C is induced by hemin treatment, cell surface proteins of N2a5E4E cells were pulse-labeled with biotin for 5 min, incubated with or without hemin (10 μM) for 1 h to allow for turnover, and isolated on streptavidin beads for immunoblot analysis. With hemin treatment, the biotinylated PrP^C bands decreased compared with those in untreated cells, whereas the overall banding patterns and intensity of other biotinylated proteins was not noticeably affected (Fig. 5, a and b). These results clarify that the decrease of PrP^C signal in response to hemin treatment is not because of reduced expression but to enhanced degradation.

Hemin effects on the biosynthesis and turnover of PrP^C were also evaluated by pulse-chase [35S]methionine labeling and radioimmunoprecipitation of PrP^C in human NB1 cells that express endogenous levels of PrP^C. At time 0, immature glycosylated and unglycosylated forms of PrP^C were seen as described previously (Fig. 5c, arrows) (35). After a 1-h chase, mature glycosylated forms predominated (Fig. 5c, asterisk). In hemin (10 μM)-treated cells, these PrP^C bands disappeared more rapidly with increasing chase periods, showing increased PrP^C turnover relative to that seen in control cells. The quantification of all glycosylated and unglycosylated PrP^C bands from two experiments revealed that the loss of pulse-labeled PrP^C was accelerated in the presence of 10 μM hemin (Fig. 5d). Altogether, these data show that hemin selectively alters the subcellular localization and turnover of the PrP^C.

PrP^C Binding to Hemin-Agarose—To evaluate whether hemin can directly interact with PrP^C, hemin-agarose affinity chromatography was performed using N2a5E4E and NB1 cell lysates. Amounts of PrP^C in bound and unbound fractions were analyzed by Western blotting. A single aliquot of hemin-agarose beads was able to fractionate ~50% of PrP^C from the total cell lysate (Fig. 6, a and b, lane 2). Additional PrP^C (~25%) could be extracted from the lysate with a fresh aliquot of hemin-agarose beads (data not shown). The absence of PrP^C binding to agarose beads without hemin confirmed the specificity of the interaction between hemin and PrP^C (Fig. 6, a and b, lane 4). To assess the selectivity of PrP^C binding, the other proteins of each fraction were stained nonspecifically with GelCode Blue. A number of other proteins from the cell lysates also bound to hemin-agarose, as expected, but most proteins were much more abundant in the unbound fraction (Fig. 6, a and b, lanes 5 and 6). Thus, the hemin-agarose showed some selectivity for binding PrP^C. As an additional indication of specificity and to examine whether the octapeptide repeats in PrP^C might be involved in hemin binding, we evaluated the hemin-agarose binding of hamster PrP^C lacking the octapeptide repeats and flanking sequences (HaPrP Δ34–94). Although the binding of wild-type hamster PrP^C was as efficient as the binding of the wild-type mouse and human PrP^C, only ~10% of HaPrP Δ34–94 bound to hemin-agarose (Fig. 6c). These results showed that hemin interacts directly or indirectly with PrP^C of multiple species and that PrP^C residues 34–94 strongly influence that interaction.

UV-visible Spectroscopy—To obtain additional evidence of direct interactions between hemin and PrP^C, we used UV-visible spectroscopy. Hemin is sparingly soluble in aqueous media and, when not bound to proteins, tends to form oligomers that absorb strongly at ~390 nm (the Soret band). This absorbance maximum (A_{max}) can shift to different wavelengths upon interaction with other molecules. Using this spectral property of hemin, we evaluated the binding of hemin to purified rPrP^C. Spectra of hemin alone at various concentrations showed that the A_{max} was slightly blue-shifted with increasing concentrations (Fig. 7, a–c, blue lines). However, when hemin was incubated with rPrP^C at a 1:1 molar ratio, the A_{max} red-shifted to
411 ± 3 nm independent of the concentration of the complex (Fig. 7a, dotted green line). These results suggested that PrP C reorganized hemin molecules into distinct oligomeric states. Similar spectral changes were produced with C-terminally truncated rPrP (residues 23–106), which contains the octapeptide repeats (Fig. 7a, dotted pink line). However, the N-terminally truncated rPrP (residues 90–231) did not alter the hemin spectrum indicating that the C-terminal residues 90–231 were not required for hemin binding (Fig. 7a, dotted orange line).

Bovine serum albumin (BSA) is a well known hemin-binding protein that has nanomolar affinity for hemin (36). When BSA interacted with hemin, only a small red shift of \(A_{\text{max}}\) (396 ± 2 nm) occurred (Fig. 7b, dotted pink line). These different effects of rPrP C and BSA on the hemin spectrum indicated that PrP C and BSA interact in distinct ways with hemin.

To determine the stoichiometry of the observed hemin-PrP C interactions, increasing concentrations of hemin were added to a fixed rPrP C concentration (Fig. 7c) and vice versa (not shown). At molar excesses of hemin up to ~10:1, the full \(A_{\text{max}}\) red shift was maintained, indicating that each PrP molecule could influence the spectrum of multiple hemin molecules. However, with further increases in the hemin:PrP C ratio, the \(A_{\text{max}}\) gradually shifted back toward the \(A_{\text{max}}\) of free hemin, suggesting that saturation of the binding to rPrP C had occurred. Taken together, these data indicate that multiple hemin molecules can bind directly to PrP C, primarily via the N-terminal half of the molecule.

**Enhancement of the Peroxidase Activity of Hemin by Interactions with rPrP C**—Given the observed interactions between hemin and PrP C, we sought clues as to whether such interactions might have additional physiological significance. It has been reported that an excess of free hemin can have cytolytic activity because of its inherent peroxidase activity (37). To see if binding to PrP C might alter such activities of hemin, we compared the peroxidase activity of free hemin and its rPrP C complex. In an assay using TMB as a substrate, the hemin-rPrP C complex showed increased peroxidase activity by up to 3-fold compared with hemin alone (Fig. 8). Similar results were also obtained using 2,2’-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt as a substrate (data not shown). Consistent with previous studies (36), the binding of hemin to BSA also increased its peroxidase activity (Fig. 8). In contrast, no superoxide dismutase or catalase activities of hemin itself or hemin-rPrP C complexes were observed (data not shown). The
fact that the peroxidase activity of hemin is altered by binding to PrP<sub>C</sub> indicates that the interaction affects the inherent redox properties of this porphyrin.

**DISCUSSION**

Hemin interacts with a number of proteins stably or reversibly and orchestrates various vital biological activities. Here we have demonstrated that PrP<sub>C</sub> is a hemin-binding protein that undergoes aggregation, internalization, and degradation upon exposure to hemin.

**Potential Relevance of Hemin Binding and Cellular Trafficking in Biological Activities of PrP<sub>C</sub>**—PrP<sub>C</sub> constitutively cycles between the plasma membrane and endocytic compartments, and its endocytosis can take place via a clathrin-dependent mechanism (38, 39). Copper, at very high concentrations (100 μM), can enhance the endocytosis of PrP<sub>C</sub>, leading to proposals that it functions as a transporter or sensor for metal ions (18, 40). The internalization of PrP<sub>C</sub> is also involved in nitric oxide-dependent autoprocessing of glypican-1 (41) and in p53-dependent staurosporine-induced caspase-3 activation (42, 43). These observations suggest that cellular trafficking of PrP<sub>C</sub> is closely related to its physiological activities. Thus, the fact that hemin binding alters the PrP<sub>C</sub> trafficking suggests that PrP<sub>C</sub> may participate in hemin-dependent biological events and/or that hemin binding is relevant in PrP<sub>C</sub> functions.

The endocytosis of PrP<sub>C</sub> through clathrin-coated pits requires a transmembrane receptor. Recently, the low density lipoprotein receptor-related protein was identified as the transmembrane receptor for PrP<sub>C</sub> binding to hemin-agarose. a, N2a5E4E cell lysates were incubated with hemin-agarose (Hm-AG) or agarose-only (AG) beads. The amounts of PrP<sub>C</sub> or other proteins in bound (B) and unbound (U) fractions were assessed by immunoblotting for PrP<sub>C</sub> and GelCode Blue staining of total proteins. b and c, same procedure was performed using NB1 cells that express human PrP<sub>C</sub> (b) and CF10 cells that express full-length hamster PrP<sub>C</sub> (HaPrP<sub>C</sub>) or HaPrP<sub>C</sub> lacking residues 34–94 (c). The data are representative of the results of two independent binding experiments performed in each cell type.

**FIGURE 6. PrP<sub>C</sub> binding to hemin-agarose.** a, N2a5E4E cell lysates were incubated with hemin-agarose (Hm-AG) or agarose-only (AG) beads. The amounts of PrP<sub>C</sub> or other proteins in bound (B) and unbound (U) fractions were assessed by immunoblotting for PrP<sub>C</sub> and GelCode Blue staining of total proteins. b and c, same procedure was performed using NB1 cells that express human PrP<sub>C</sub> (b) and CF10 cells that express full-length hamster PrP<sub>C</sub> (HaPrP<sub>C</sub>) or HaPrP<sub>C</sub> lacking residues 34–94 (c). The data are representative of the results of two independent binding experiments performed in each cell type.

**FIGURE 7. Modification of absorption spectra of hemin by recombinant PrP<sub>C</sub>.** a, hemin absorption spectrum is represented by the solid blue line. The addition of equimolar concentrations of either full-length PrP<sup>C</sup> (PrP residues 23–231; dotted green line) or C-terminally truncated rPrP (PrP residues 23–106; dotted pink line) red-shifted the A<sub>max</sub> of free hemin, whereas N-terminally truncated rPrP (PrP residues 90–231; dotted orange line) did not alter the hemin spectrum. The numbers indicate the mean of the wavelength in nm at the A<sub>max</sub>. b, A<sub>max</sub> of the hemin spectrum (solid blue line) was slightly redshifted (dotted pink line) by interactions with BSA. c, the red shift of the hemin spectrum was maintained in the presence of rPrP at molar excesses of hemin up to 10:1 (compare green lines with the yellow and orange lines). Further molar excesses of hemin gradually reduced the red shift of the hemin A<sub>max</sub> (pink and brown lines). For easier comparison to the spectra at highest hemin: PrP ratios, the spectrum of 4:1 hemin:rPrP residues 23–231 (solid blue line) was multiplied by a factor of 5 (dotted yellow line). The spectra shown are representative of spectra of at least four replicate samples.
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FIGURE 8. PrP<sup>C</sup> binding increases the inherent peroxidase activity of hemin. Hemin and rPrP<sup>C</sup> were mixed prior to reaction with the TMB substrate, and the reaction was monitored by absorbance at 650 nm as a function of time. Hemin (8 μM) alone had some activity (●), and this activity was enhanced in the presence of 8 μM rPrP<sup>C</sup> (■). BSA (8 μM) also increased the peroxidase activity of hemin (▲). BSA (▲) and rPrP<sup>C</sup> (□) alone had no activity. The data points show the mean ± S.D. (n = 4).

receptor that mediates copper-induced PrP<sup>C</sup> internalization (44). Low density lipoprotein receptor-related protein also participates in the internalization of the hemin-hemopexin complex resulting in cellular hemin uptake (45). At the moment, it is not clear whether PrP<sup>C</sup> acts as a receptor for free hemin or interacts with hemin-hemopexin complexes and acts as a co-receptor in compartments such as blood and liver when these complexes are formed. However, given that both copper and hemin are small ligands that have redox properties and appear to bind a similar region of PrP<sup>C</sup>, it is possible that both of these ligands induce the internalization of PrP<sup>C</sup> by a related mechanism. Further studies will be required to evaluate this possibility.

PrP<sup>C</sup> trafficking also influences its conversion into PrP<sup>Sc</sup> and disruption of the normal trafficking of PrP<sup>Sc</sup> seems to be a common mechanism for several classes of PrP<sup>Sc</sup> inhibitors (23, 46). Notably, the hemin concentration that effectively altered PrP<sup>C</sup> trafficking (~3 μM) also reduced the formation of PrP<sup>Sc</sup> in scrapie-infected cell cultures (data not shown). Therefore, the anti-scrapie activity of hemin in cell culture models might relate to its ability to stimulate the internalization of PrP<sup>C</sup>.

Roles of Peroxidase Activity of the Hemin-PrP<sup>C</sup> Complex—Hemin-containing peroxidases react with H<sub>2</sub>O<sub>2</sub> and promote oxidative modifications of proteins, lipids, and halides (47). It is well known that hemin forms oligomers in aqueous media. The fact that co-incubation of hemin with rPrP<sup>C</sup> caused a red shift in the Soret region of the UV-visible spectrum indicates that hemin oligomers reorganize in the presence of PrP<sup>C</sup>. As a result, they become more reactive with H<sub>2</sub>O<sub>2</sub> as indicated by the PrP<sup>C</sup>-induced enhancement of the peroxidase activity of hemin (Fig. 8).

The activity of antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase, is altered in PrP<sup>C</sup> null mice and in scrapie-infected brains (48, 49). These findings suggest that PrP<sup>C</sup> participates in the modulation of the activity of these enzymes and/or PrP<sup>C</sup> itself functions as an antioxidant molecule. Moreover, it has been reported that the flexible N-termin-
