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Crosslinking of hemin to a specific site on the 90-kDa ferritin repressor protein

(iron/translational regulation/induction of gene expression)

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ABSTRACT **Incubation of a 90-kDa ferritin repressor protein (FRP) with small amounts of radiolabeled hemin resulted in the formation of a strong interaction between the two that was stable to SDS/PAGE. (We refer to this interaction as a "crosslink," without intending to imply knowledge as to its chemical nature.) Of seven other proteins tested individually, only apohemopexin and bovine serum albumin showed similar crosslinking ability, albeit to a much lower extent. [^{14}C]Hemin specifically crosslinked to FRP in the presence of a 50-fold excess of total wheat germ proteins. Inclusion of catalase did not prevent the reaction of hemin with FRP, suggesting that H_2O_2 is not involved. The subsequent addition of a stoichiometric amount of apohemopexin did not reverse the reaction. Exhaustive digestion of the complex with *Staphylococcus aureus* V8 protease produced a major labeled peptide of 17 kDa. These results show the existence of a highly specific, uniquely reactive hemin binding site on FRP.**

Synthesis of ferritins, the iron-storage proteins, is regulated primarily at the translational level (1–8). In the absence of extracellular iron, a 90-kDa ferritin repressor protein (FRP) binds to a 28-nucleotide iron-responsive element (IRE) in the 5′ untranslated region of the ferritin mRNA (9–18). This prevents translation by ribosomes (9–11). In the presence of extracellular iron, the FRP is inactivated (19, 20) and translation proceeds uninhibited. Derepression of ferritin synthesis can also be observed *in vitro*, where hemin acts as a highly specific inducer (21, 22). It was inferred from these *in vitro* experiments that one or more specific binding sites for hemin must exist on the FRP. Here we show that this is the case. Surprisingly, hemin forms a stable, possibly covalent, crosslink to some element of this binding site on FRP. Since recent results from our laboratory indicate that heme, or a closely related molecule, can also induce ferritin synthesis *in vivo* (23), we suggest that this process may occur via a direct binding of hemin to FRP, which inactivates FRP for repression and allows translation of the ferritin mRNA to proceed unimpeded. It is possible, although not necessary, that these events involve formation of a covalent bond between hemin and FRP.

MATERIALS AND METHODS

[^{14}C]Hemin (Leeds Radioporphyrins, Leeds, U.K.; 97 Ci/mol; 1 Ci = 37 GBq) was purchased in aliquots of 1 μCi. Aqueous solutions were stable for only short periods even when stored at −70°C. The reaction between >95% pure FRP (0.7 μg) and hemin (5–50 μM) was conducted in a 4-μl volume containing 20 mM Hepes/KOH (pH 7.4), 100 mM KCl, 5% (vol/vol) glycerol, 0.05 mM EDTA, and other components as indicated in the figure legends. These conditions are similar to the preincubation step previously described (21), except that small molecules were first removed from all proteins by Centricon 30 (Amicon) ultrafiltration, the glutathione redox buffer was omitted, and the reaction time was 60 min at 37°C (maximum crosslinking was ordinarily achieved in 30–60 min). When excess wheat germ proteins were present, reaction times were varied from 15 min (as shown in Fig. 3) to 60 min in order to detect differential rates of crosslinking to different proteins. No such effects were seen. Reaction products were analyzed by SDS/10% PAGE and fluorography.

The stoichiometry of the crosslinking reaction was determined by excising labeled bands from the gel shown in Fig. 2 and allowing the gel slices to swell overnight in Protosol (NEN), before assay of radioactivity in 3A70 scintillation fluid (RPI, Mount Prospect, IL).

RESULTS AND DISCUSSION

Evidence for the crosslinking of hemin to FRP was obtained by incubating freshly prepared [^{14}C]hemin with highly purified FRP or other proteins and analyzing the products by SDS/PAGE and fluorography. Typical results are shown in Fig. 1. It is evident that the amount of ^{14}C associated with FRP increased with hemin concentration and that other proteins present in the reaction mixture, such as glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase, were unreactive with hemin. Inclusion of 2-mercaptoethanol slightly stimulated the crosslinking of hemin to FRP, most notably at 50 μM [^{14}C]hemin. In other experiments, dithiothreitol was found to be much more stimulative than 2-mercaptoethanol, although it also promoted the nonspecific labeling of virtually any protein (J.-J.L. and R.E.T., unpublished work). This is not surprising in view of the high reactivity of ferrous iron or heme that is produced by reaction with dithiothreitol (24–27). Similarly, the high concentration of H_2O_2 previously reported to facilitate the crosslinking of hemin to myoglobin (28) caused extensive nonspecific crosslinking of hemin to all proteins present (Fig. 1), as well as formation of protein–protein dimers and oligomers (29). To minimize these nonspecific reactions, all samples were extensively dialyzed prior to use in subsequent experiments.

In view of the extensive, nonspecific crosslinking catalyzed by dithiothreitol, the fact that FRP was labeled in the absence of an exogenous thiol suggested that one or more cysteine residues of the protein itself might be involved. Indeed, prior or concurrent treatment with *N*-ethylmaleimide at high concentrations reduced the labeling of FRP by 60–

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Abbreviations: FRP, ferritin repressor protein; IRE, iron-responsive element.

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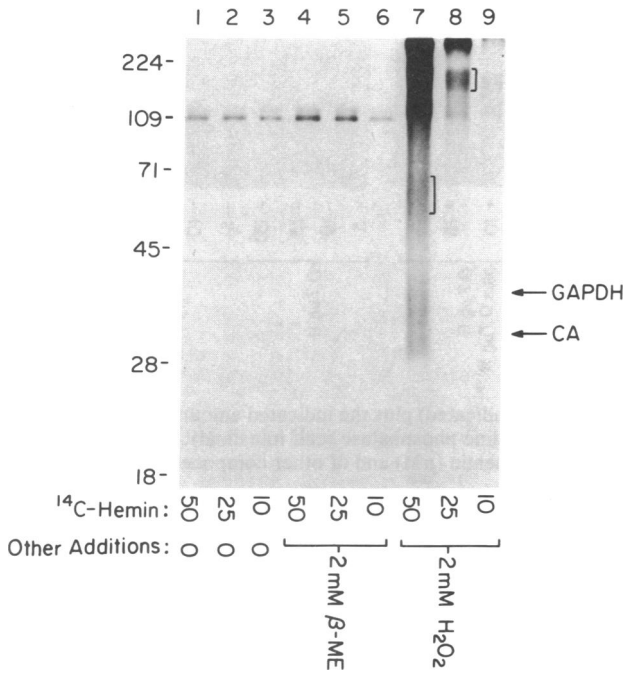

FIG. 1. Crosslinking of [^{14}C]hemin to FRP. FRP (0.7 μg; 2.0 μM) plus the indicated amounts of other reagents were incubated 60 min at 37°C and analyzed by SDS/PAGE and fluorography. All reactions contained, in addition to FRP, equimolar amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 0.28 μg) and carbonic anhydrase (CA, 0.22 μg) plus 2 mM 2-mercaptoethanol (β-ME) or 2 mM H_2O_2 as indicated. The [^{14}C]hemin concentration (μM) is shown immediately below each lane. Regions containing protein dimers are indicated by brackets. Molecular size markers (kDa) are at left.

70%, although diamide had only a modest effect (Table 1, Exp. 1). Thus, a reactive cysteine that is partially shielded from *N*-ethylmaleimide could well be involved, although residue(s) other than cysteine may also participate in the crosslinking of hemin to FRP.

A relatively high proportion of FRP molecules are capable of crosslinking: at 10 and 20 μM [^{14}C]hemin, concentrations that derepress translation by ≈10% and ≈20% of the maximum range (21), ≈8% and ≈16% of FRP molecules, respectively, were bound irreversibly to hemin. FRP that was inactivated for repression of ferritin mRNA translation by storage in the absence of reducing agents was incapable of crosslinking to [^{14}C]hemin, suggesting that an active conformation of the protein molecule is necessary for the crosslinking reaction. A similar conclusion is suggested by the fact that [^{14}C]hemin sequestered by hemopexin did not crosslink to FRP when it was released by treatment with SDS and 2-mercaptoethanol prior to electrophoresis (Table 1, Exp. 4).

Crosslinking of FRP to [^{14}C]hemin was slightly inhibited by the inclusion of other proteins, such as calf intestinal phosphatase, cytochrome *c*, apohemopexin, bovine serum albumin, or catalase (Fig. 2 and Table 1, Exp. 2 and 3). The inhibition of hemin–FRP crosslinking by the first two proteins is presumably due to their ability to bind hemin nonspecifically. This may be due to the amphipathic character of hemin, which would give it detergent activity. Of the seven proteins tested, only apohemopexin and bovine serum albumin showed traces of labeling with hemin under the conditions employed (Fig. 2, lanes 10 and 14). This is consistent with the fact that both of these proteins bind hemin specifically.

Increasing the concentrations of apohemopexin and bovine serum albumin shown in Fig. 2 by a factor of 4 (i.e., to 11–12 μM) reduced the labeling of FRP with 10 μM [^{14}C]hemin by ≈90% and ≈15%, respectively, presumably by competitive binding of hemin (Table 1, Exp. 4). This result suggests that

Table 1. Effects of other components on crosslinking of [^{14}C]hemin to FRP

Exp.	Addition(s) or change(s)	^{14}C linked to FRP, %
1	None	100
	N-ethylmaleimide	
	0.1 mM	68
	0.3 mM	34
	1.0 mM	34
	3.0 mM	35
	6.0 mM	34
	Diamide	
	3.0 mM	66
	10.0 mM	64
2	None	100
	Alkaline phosphatase (0.35 μg, 0.97 μM)	53
	Cytochrome *c* (0.7 μg, 12.5 μM)	56
	Apohemopexin (0.7 μg, 2.9 μM)	55
	Bovine serum albumin (0.7 μg, 2.7 μM)	117
	Catalase (0.7 μg, 2.9 μM)	47
	Apohemopexin + catalase (each at 2.9 μM)	16
	Hydrogen peroxide	
	100 μM	111
	500 μM	101
3*	None	100
	Alkaline phosphatase (0.35 μg, 0.97 μM)	60
	Cytochrome *c* (0.7 μg, 12.5 μM)	85
	Apohemopexin (0.7 μg, 2.9 μM)	102
	Bovine serum albumin (0.7 μg, 2.7 μM)	103
	Catalase (0.7 μg, 2.9 μM)	78
	Apohemopexin + catalase (each at 2.9 μM)	56
4	None	100
	Alkaline phosphatase (0.7 μg, 1.9 μM)	84
	Cytochrome *c* (0.7 μg, 12.5 μM)	64
	Apohemopexin (2.8 μg, 11.7 μM)	9
	Bovine serum albumin (2.8 μg, 10.6 μM)	84
5	None	100
	Incubation shortened to 15 min	61
	After 15-min incubation, apohemopexin (11.7 μM) was added; incubation then continued for 15 min	54
	After 15-min incubation, apohemopexin (11.7 μM) was added; incubation then continued for 30 min	73

Reaction conditions were the same as in lane 2 of Fig. 2, with 2.0 μM (0.7 μg) FRP and 10 μM [^{14}C]hemin, unless otherwise noted. Relative radioactivity linked to FRP was determined by densitometric scans of autoradiograms similar or identical to those shown in Fig. 2 and is expressed as a percentage of the control value obtained with no additions other than [^{14}C]hemin and FRP.
*[^{14}C]Hemin concentration was 20 μM.

the affinity of FRP for hemin lies between those of apohemopexin ($K_d \approx 10^{-12}$ M) and bovine serum albumin ($K_d \approx 10^{-6}$ M) (30–32). By contrast, addition of apohemopexin after the hemin–FRP complex had already formed did not destabilize it (Table 1, Exp. 5). Similarly, extraction with 2-butanone or precipitation with trichloroacetic acid failed to dissociate [^{14}C]hemin from the complex with FRP. These results suggest that the binding of hemin to FRP is extremely tight and are consistent with the possibility that the hemin–FRP crosslink detected by SDS/PAGE forms soon after binding.

Consistent with the lack of inhibition by catalase (Table 1, Exp. 3), low concentrations of H_2O_2 had little effect on the labeling of FRP, other than to cause the formation of slower migrating species (Fig. 2, lanes 31 and 32), which were presumably dimers and oligomers of FRP as noted above. This dimerization reaction was greatly enhanced by higher hemin and H_2O_2 concentrations (Fig. 1, lanes 7–9).

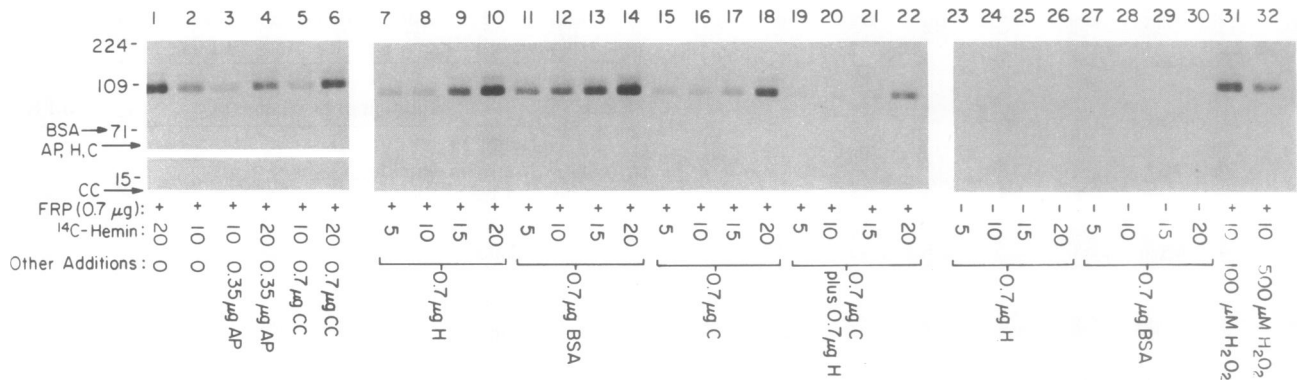

FIG. 2. Specificity of crosslinking of [^{14}C]hemin to FRP. FRP (0.7 μg, except where indicated) plus the indicated amounts of other reagents were incubated 60 min at 37°C and analyzed by SDS/PAGE and fluorography. AP, alkaline phosphatase (calf intestinal); CC, cytochrome *c*; H, apohemopexin; BSA, bovine serum albumin; C, catalase. The concentration of [^{14}C]hemin (μM) and of other components in each reaction mixture is indicated.

To see whether the hemin–FRP crosslinking reaction would occur specifically in a crude cell lysate, FRP was mixed with a large excess of wheat germ lysate protein and then incubated with 20 or 50 μM [^{14}C]hemin. Even in the presence of a 50-fold excess of cellular proteins, FRP specifically crosslinked with [^{14}C]hemin (Fig. 3). The presence of wheat germ proteins muted the crosslinking to FRP at 20 μM hemin, but at 50 μM hemin this inhibitory effect was less evident. This observation is consistent with the hemin concentration dependence of the derepression reaction previously reported (21). Only a few other wheat germ proteins appeared to react significantly with hemin. These were probably heme-binding proteins. Thus, the interaction between FRP and hemin is highly specific.

To map the site of hemin linkage to FRP, crosslinked complex was formed, and then the reaction mixtures were digested with *Staphylococcus aureus* V8 protease. Under these conditions (Hepes buffer, pH 7) the hemin–FRP complex was relatively resistant to digestion (Fig. 4*A*). However, with equal amounts of protease and FRP, a prominent radioactive band of ≈17 kDa was produced. No other FRP peptide, as visualized by silver staining, was labeled with [^{14}C]hemin (compare lanes 1 and 4 of Fig. 4*B*; under these conditions V8 protease completely digests itself, as shown in lane 2 of Fig. 4*B*). These observations confirm that hemin crosslinks to a unique site on the FRP molecule.

Covalent crosslinking of heme to amino acid residues occurs naturally with a number of stable heme proteins, notably the cytochromes. Myoglobin has been shown to crosslink with heme (28, 33), although high concentrations of H_2O_2 (2 mM) or an alkyl halide (3 mM) are required for this reaction. Thus, the crosslinking between FRP and hemin described here appears to be unique. Whether this crosslinking reaction is an obligatory intermediate in the derepression process or is only a side reaction cannot be determined at present. Moreover, we have no evidence as to whether it occurs *in vivo*. However, at the very least, this reaction provides a convenient method for studying the specific binding site for hemin on FRP. It has been argued elsewhere

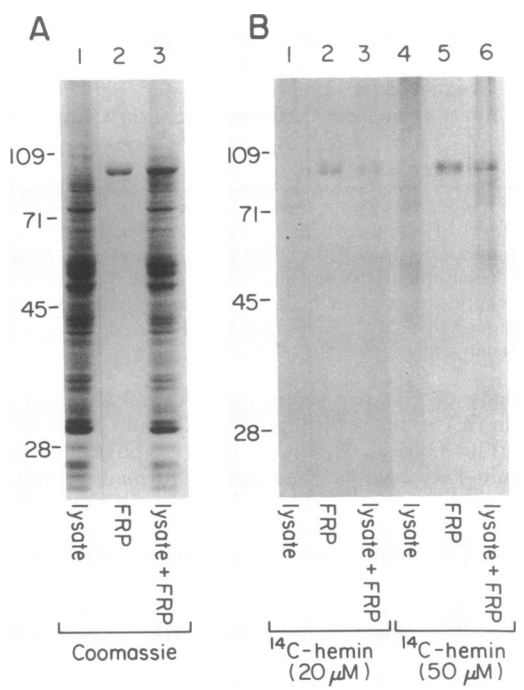

FIG. 3. Crosslinking of [^{14}C]hemin to FRP in the presence of wheat germ lysate proteins. FRP (0.7 μg) plus wheat germ lysate (35 μg) were incubated 15 min at 37°C with 20 or 50 μM [^{14}C]hemin and analyzed by SDS/PAGE and fluorography (*B*). Equivalent samples of these proteins were analyzed by SDS/PAGE and stained with Coomassie blue (*A*).

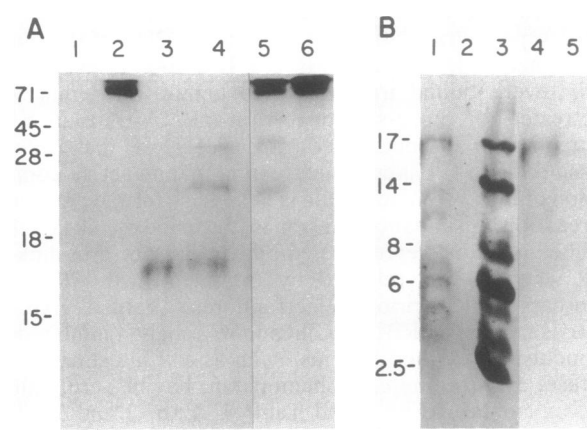

FIG. 4. Mapping of FRP peptides labeled with [^{14}C]hemin by digestion with *S. aureus* V8 protease. FRP (0.7 μg, except where indicated) was incubated with 10 μM [^{14}C]hemin for 60 min. The indicated amounts of protease were then added and the reaction was continued overnight. Labeled products were analyzed by gel electrophoresis and fluorography (*A*, lanes 1–6; *B*, lanes 4 and 5) or silver staining (*B*, lanes 1–3). (*A*) Lane 1, no FRP, 0.7 μg of protease; lane 2, no protease; lane 3, 0.7 μg of protease; lane 4, 0.2 μg of protease; lane 5, 0.07 μg of protease; lane 6, 0.015 μg of protease. (*B*) Lane 1, 0.7 μg of protease (plus 0.7 μg of [^{14}C]hemin–FRP adduct), silver-stained; lane 2, same as lane 1, but lacking [^{14}C]hemin–FRP adduct; lane 3, peptide markers, silver-stained; lane 4, fluorogram of lane 1; lane 5, fluorogram of lane 2.

that a total hemin concentration of 10 μM *in vitro* corresponds to a monomeric or "free" hemin concentration of <200 nM, due to the tendency of hemin to aggregate (22), and that the hemin monomer is probably the active form in binding to FRP. Thus, both the crosslinking of FRP to hemin and the inactivation of FRP by hemin may occur at a physiological hemin concentration.

The mechanism by which hemin binding and/or crosslinking reduces the affinity of the FRP for the IRE remains unclear. Models in which the hemin and IRE binding sites partially or completely overlap, or in which an allosteric change in one site is induced by occupancy of the other, or in which the interaction between the two sites is mediated by a "sulfhydryl switch" (13, 14, 19) all seem possible at present. Other interesting questions involve the detailed chemistry of the binding and crosslinking reactions, and whether complexes of iron other than hemin can react at this same site.

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1. Munro, H. N. & Linder, M. C. (1978) *Physiol. Rev.* **58,** 317–396.
2. Aisen, P. & Listowsky, I. (1980) *Annu. Rev. Biochem.* **49,** 357–393.
3. Theil, E. C. (1987) *Annu. Rev. Biochem.* **56,** 289–315.
4. Drysdale, W. & Munro, H. N. (1966) *J. Biol. Chem.* **241,** 3630–3637.
5. Chu, L. L. H. & Fineberg, R. A. (1969) *J. Biol. Chem.* **244,** 3847–3854.
6. Zahringer, J., Baliga, B. S. & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 857–861.
7. Shull, G. E. & Theil, E. C. (1982) *J. Biol. Chem.* **257,** 14187–14191.
8. Aziz, N. & Munro, H. N. (1986) *Nucleic Acids Res.* **14,** 915–927.
9. Walden, W. E., Daniels-McQueen, S., Brown, P. H., Gaffield, L., Russell, D. A., Bielser, D., Bailey, L. C. & Thach, R. E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9503–9507.
10. Brown, P. H., Daniels-McQueen, S., Walden, W. E., Patino, M. M., Gaffield, L., Bielser, D. & Thach, R. E. (1989) *J. Biol. Chem.* **264,** 13383–13386.
11. Walden, W. E., Patino, M. M. & Gaffield, L. (1989) *J. Biol. Chem.* **264,** 13765–13769.
12. Liebold, E. E. & Munro, H. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2171–2175.
13. Rouault, T. A., Hentze, M. W., Caughman, S. W., Harford, J. B. & Klausner, R. D. (1988) *Science* **241,** 1207–1210.
14. Hentze, M. W., Rouault, T. A., Harford, J. B. & Klausner, R. D. (1989) *Science* **244,** 357–359.
15. Hentze, M. W., Rouault, T. A., Caughman, S. W., Dancis, A., Harford, J. B. & Klausner, R. D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6730–6734.
16. Murray, M. T., White, K. & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7438–7442.
17. Hentze, M. W., Caughman, S. W., Rouault, R. A., Barriocanal, J. G., Dancis, A., Harford, J. B. & Klausner, R. D. (1987) *Science* **238,** 1570–1573.
18. Aziz, N. & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8478–8482.
19. Haile, D. J., Hentze, M. W., Rouault, T. A., Harford, J. B. & Klausner, R. D. (1989) *Mol. Cell. Biol.* **9,** 5055–5061.
20. Swenson, G. S., Patino, M. M., Beck, M. M., Gaffield, L. & Walden, W. E. (1991) *Biol. Metals* **4,** 48–55.
21. Lin, J. J., Daniels-McQueen, S., Patino, M. M., Gaffield, L., Walden, W. E. & Thach, R. E. (1990) *Science* **247,** 74–77.
22. Lin, J. J., Patino, M. M., Gaffield, L., Walden, W. E. & Thach, R. E. (1990) *Biochim. Biophys. Acta* **1050,** 146–150.
23. Goessling, L. S., Lin, J. J. & Thach, R. E. (1991) *FASEB J.* **5,** A1539 (abstr.).
24. Kim, K., Rhee, S. G. & Stadtman, E. R. (1985) *J. Biol. Chem.* **260,** 15394–15397.
25. Vincent, S. H. & Muller-Eberhard, U. (1987) *J. Lab. Clin. Med.* **110,** 475–482.
26. Vincent, S. H., Grady, R. W., Shaklai, N., Snider, J. M. & Muller-Eberhard, U. (1988) *Arch. Biochem. Biophys.* **265,** 539–550.
27. Aft, R. L. & Mueller, G. C. (1985) *J. Biol. Chem.* **259,** 301–305.
28. Catalano, C. E., Cheo, Y. S. & Ortiz de Montellano, P. R. (1989) *J. Biol. Chem.* **264,** 10534–10541.
29. Tew, D. & Ortiz de Montellano, P. R. (1988) *J. Biol. Chem.* **263,** 17880–17886.
30. Sinclair, P. R., Bement, W. J., Gorman, N., Liem, H. H., Walkoff, A. W. & Muller-Eberhard, U. (1988) *Biochem. J.* **256,** 159–165.
31. Smith, A. (1990) in *Biosynthesis of Heme and Chlorophylls*, ed. Dailey, H. (McGraw-Hill, New York), pp. 435–490.
32. Toenberg, M. & Margalit, R. (1985) *Biochem. J.* **229,** 197–203.
33. Osawa, Y., Martin, B. M., Griffin, P. R., Yates, J. R., Shabanowitz, J., Hunt, D. F., Murphy, A. C., Chen, L., Cotter, R. J. & Pohl, L. R. (1990) *J. Biol. Chem.* **265,** 10340–10346.