Identification of a Heme-sensing Domain in Iron Regulatory Protein 2*

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Iron regulatory protein 2 coordinates the cellular regulation of iron metabolism by binding to iron-responsive elements in mRNA. The protein is synthesized constitutively but is rapidly degraded when iron stores are replete. The mechanisms that prevent degradation during iron deficiency or promote degradation during iron sufficiency are not delineated. Iron regulatory protein 2 contains a domain not present in the closely related iron regulatory protein 1, and we found that this domain binds heme with high affinity. A cysteine within the domain is axially liganded to the heme, as occurs in cytochrome P450. The protein-bound heme reacts with molecular oxygen to mediate the oxidation of cysteine, including β-elimination of the sulfur to yield alanine. This covalent modification may thus mark the protein molecule for degradation by the proteasome system, providing another mechanism by which heme can regulate the level of iron regulatory protein 2.

Iron metabolism is exquisitely regulated by all organisms, from bacteria to humans. In mammals, the iron regulatory proteins (IRPs)1 mediate the coordinate expression of proteins that participate in iron metabolism (1, 2). When iron stores are low, the IRPs bind to an RNA stem-loop structure known as an iron-responsive element (IRE) located in either the 5′- or 3′-untranslated region of mRNA. If the IRE is close to the cap site, binding of the IRP blocks the initiation of translation, causing a decrease in the level of the protein encoded by that mRNA. Conversely, when the IRE is located in the 3′-untranslated region of the transferrin receptor transcript, binding of the IRP stabilizes the mRNA by decreasing susceptibility to nuclease attack, causing an increase in the level of the protein encoded by the mRNA (3).

Mammals have two known IRPs, IRP1 and IRP2, with the tissue roles of each still being defined. The general mechanism by which each IRP is regulated is distinct (1). When cellular iron stores are low, IRP1 lacks a functional iron-sulfur center and binds to its IRE targets. When iron stores are sufficient, IRP1 regains its full iron-sulfur center, loses the ability to bind to IRE, and functions as a cytosolic aconitase. The cellular levels of IRP1 are unaffected by iron status in most cell types.

In contrast, the IRP2 protein and IRE binding activity are readily detected when iron stores are limited but are low or absent when iron stores are sufficient (4, 5). The decrease in the IRP2 protein occurs as a consequence of rapid degradation by the proteasome; synthesis of the protein is constitutive and does not vary substantially with iron status. The sequences of the two IRPs are similar except that IRP2 contains one domain not present in IRP1. In addition to its absence from IRP1, the sequence of this 73-residue domain is not homologous to any sequences in the current National Center for Biotechnology Information (NCBI) data bases. Initial studies of the domain suggested that it was required to confer susceptibility to iron-dependent degradation, and it has thus been referred to as the “degradation domain” (6). Subsequent studies from several laboratories have established that the domain is not absolutely required for iron-triggered degradation, suggesting that there may be multiple mechanisms by which degradation can occur (7–9). Cys-178 within the domain is required for degradation triggered by sodium nitroprusside (10).

In a recent study of the recombinant 63-residue domain, we showed that it can act as an iron sensor, mediating its own covalent modification (11). The domain forms an iron-binding site with three cysteine residues located in the middle of the domain. The protein-bound iron then reacts with molecular oxygen to generate an oxidizing species at the iron-binding site. One cysteine residue is oxidized to dehydrocysteine and other products. We suggested that this oxidative modification may be one mechanism for marking the molecule for degradation by the proteasome system.

Redox-cycling cations such as iron and copper are capable of catalyzing the generation of reactive oxygen species through a Fenton reaction and other types of reactions. Consequently, the concentrations of free iron and copper in the cell are held at very low levels, with their transport carefully orchestrated by a series of chaperones (12, 13). Furthermore, >75% of the body’s iron content is in the form of heme (14), and, thus, a sensor that detects cellular heme levels would effectively be an iron sensor. Several investigators have presented evidence suggesting that IRP2 levels are sensitive to heme levels (8, 15, 16). Heme functions as a regulator of protein function in a variety of pathways from microorganisms to mammals. For example, heme regulates the iron-dependent degradation of a bacterial iron response regulator (17), and it also regulates the import of δ-aminolevulinate synthetase into mitochondria (18). We therefore investigated the possibility that the novel domain in IRP2 might function as a heme sensor.

EXPERIMENTAL PROCEDURES

Most of the procedures and sources of material were as described in our study on the iron-mediated oxidative modification of IRP2 (11). We obtained hemin chloride, protoporphyrin IX, and zinc-protoporphyrin IX from ICN Biomedicals (Irvine, CA). Hemin was dissolved to 5 mM in 100 mM NaOH, then diluted in water, and finally introduced into the...
incubation buffer, the pH of which was not changed by the addition of hemin. Solutions were made fresh daily. Sequencing grade porcine trypsin was from Promega (Madison, WI). Peptide digestion, reverse phase chromatography, and mass spectrometric sequencing was carried out as described (11), except that Lys-C was replaced by trypsin at a 1:20 ratio of enzyme/peptide. Digestion was in 1 mM Tris, pH 8.5, at 37 °C for 12–13 h.

Except as noted under “Results,” oxidation by heme was carried out in 50 mM Hapes, pH 7.2, with 2 mM TCEP and 1 mM diethylenetriaminepentaacetic acid at 37 °C. Spectrophotometric binding studies were carried out with solutions of heme and peptide that were first made anaerobic by room temperature incubation for 15 min in a chamber with an atmosphere of 97% nitrogen, 3% hydrogen, and <1 ppm oxygen (Coy Laboratory Products model 7500-000, Grass Lake, MI). UV-visible difference spectra were recorded on a Hewlett-Packard model 8452 diode array spectrophotometer, with the concentration of heme kept equal in both the sample and reference cuvettes.

The assay for heme peroxidase activity was based on the method of Vyletich and Osawa (19), modified for in-gel use. After incubation of the peptide and heme, SDS-containing sample buffer was added. This buffer contains 15 mM TCEP as a reducing agent and neither dithiothreitol nor β-mercaptoethanol. The other components are 62.5 mM Tris, pH 6.8, 2% SDS, 35% glycerol, and 0.1% bromphenol blue. Without heating, the sample was loaded onto a 16% polyacrylamide gel (Invitrogen). Following electrophoresis, the gels were soaked in a chemiluminescent peroxidase substrate for 5 min (ECL product RPN2106, Amersham Biosciences) and visualized on x-ray film (Kodak Biomax ML or MR).

RESULTS

The Domain Binds Heme—The 63-residue recombinantly expressed domain contains three cysteine residues, which, as we showed previously, are involved in the binding of ferrous iron (11). The peptide also binds heme, demonstrated by the heme difference spectrum generated when heme and peptide were present in the sample cuvette and the same concentration of heme was in the reference cuvette (Fig. 1). The observed difference spectrum is similar to that observed with cytochrome P450 (20–22). The Soret band intensity increases and splits with one peak moving to the blue (370–380 nm) and the other moving to the red (420 nm or higher). This is termed a “hyperporphyrin” spectrum (23) and is characteristic of a thiolate axially liganded to ferric heme. While the cytochrome P450s are the best-known example of such spectra, they are also seen in thiol-containing model compounds (24, 25) and in chloroperoxidase upon thiol binding (26).

For convenience we will refer to this region of IRP2 as a “heme-binding domain.” The affinity of the binding of heme is at least 10^6 M^-1 as reflected by the linear titration with heme in the micromolar concentration range^2 (Fig. 2). One mole of the heme-binding domain binds 2 mol of heme. Because heme dimerizes at concentrations of 1 μM or higher (27), we cannot distinguish between the binding of one dimerized heme and the binding of two monomeric hemos. Because the spectral changes induced by binding establish that a cysteine is an axial ligand, we examined the behavior of a mutant peptide, which we call the C123A mutant, in which all three cysteine residues (Cys-168, Cys-174, and Cys-178) were changed to alanine (Figs. 1 and 2). Heme still binds to the peptide with high affinity, but the magnitude of the difference spectrum is much less than that for wild type.

The cysteines of the peptide tend to form disulfide bonds, which we prevent by the inclusion of the phosphine TCEP in the buffer (11). Lower molecular weight phosphines are known to be able to axially ligate the heme of cytochrome P450 and induce a hyperporphyrin spectrum (24, 28). However, the phosphine was not required to induce the hyperporphyrin spectrum upon the binding of heme to the heme-binding domain of IRP2. This was determined by reducing the peptide with TCEP for 30 min and then separating the TCEP from the peptide by reverse phase chromatography. The TCEP-free peptide showed the same spectral changes as observed in the presence of TCEP.

Heme and Oxygen Mediate Covalent Modification of the Domain—We demonstrated previously that iron could mediate an oxygen-dependent covalent modification of the domain, with oxidation of up to one of the three cysteine residues in each molecule (11). Amino acid analysis and mass spectrometry were utilized to demonstrate that heme also catalyzes the oxidative modification of cysteine. To minimize possible secondary reactions, we studied the stoichiometry of peptide that was 50% saturated with heme and found that half of the cysteine residue was modified (Fig. 3). Mass spectrometric analysis (Table I) demonstrated the conversion of cysteine to several products, including the novel amino acid aminomalate, which was also observed with iron-mediated oxidation. However, with heme-mediated oxidation the quantitatively largest product observed was 32 atomic mass units less than the control peptide when analyzed without alkylation of the cysteines. Thirty-two is, of course, the mass of sulfur. When alkylated with iodoacetamide, the mass difference increased to 89 atomic mass units, implying that the product could not be alkylated.
Heme Sensing by IRP2

and accounting for the additional loss of 57 atomic mass units from the carboxyamidomethyl moiety (32 + 57 = 89). These results indicate that cysteine undergoes β-elimination of sulfur to yield alanine as the product. In some molecules, two cysteine residues were oxidized to alanine (Table I). No modification occurred when either oxygen or heme was omitted or when either zinc protoporphyrin or free protoporphyrin was substituted for heme. Also, the C123A mutant (Cys-168, Cys-174, and Cys-178) was not modified despite its ability to bind heme. The reaction was not due to adventitious free iron from the hemin or other solutions, because diethylenetriamine-pentaacetic acid was always present in the reaction solution. We demonstrated previously that diethylenetriamine-pentaacetic acid completely blocks the iron-dependent oxidation of the peptide (11).

TABLE I
Mass spectral analysis of oxidatively modified heme-binding domain

| Measured mass | Fraction | Change in mass | Assignment          |
|---------------|----------|----------------|---------------------|
| 6,694.1       | 0.66     | 0              | Native peptide      |
| 6,605.2       | 0.14     | −88.9          | 1 Cys → Ala         |
| 6,516.0       | 0.09     | −178.1         | 2 Cys → Ala         |
| 6,835.6       | 0.10     | −58.5          | 1 Cys → aminomalonic acid |

Tryptic digestion of the heme-binding domain yields a peptide mixture in which the three cysteine residues are in different peptides. Reverse phase mapping with analysis by high accuracy time-of-flight mass spectrometry established that two of the three cysteines could be oxidized to alanine (Cys-174 and Cys-178), whereas Cys-168 was not. The conversion of cysteine to alanine was confirmed by tandem mass spectrometric sequencing of the tryptic peptides (Table II).

Covalent Binding of Heme—Proteins that bind heme may undergo oxidative modifications that lead to covalent binding of the heme (29). The 63-residue heme-binding IRP2 peptide was exposed to heme under anaerobic conditions, and the unmodified peptide was completely separated from heme by reverse phase chromatography. When both heme and oxygen were present, the wild-type peptide was oxidatively modified as described above. In addition, up to 3% of the peptide covalently bound heme as determined from the area of the 400-nm high performance liquid chromatography chromatogram (30) (not shown). Although this is a minor reaction, it can easily be monitored by taking advantage of the inherent peroxidase activity of heme. When performed with a chemiluminescent substrate, the reaction provides a very sensitive assay for heme-linked proteins or peptides (19). SDS gel electrophoresis separates free heme from the peptide, allowing in-gel detection (Fig. 4). Covalent binding of heme required oxygen. Examination of the susceptibility of various cysteine mutants suggested that Cys-168 is most important for the covalent binding of heme; no covalent binding to the C123A mutant (Cys-168, Cys-174, and Cys-178) could be detected. The optimal pH for the reaction was 7.0–7.2.

The heme-binding domain has no histidine residues, an amino acid to which heme is covalently linked in many heme-containing proteins. Covalent linkage of the heme to the thiol of a cysteine was a reasonable possibility. Such thioether linkages, as occur in cytochrome c, are readily cleaved by silver or mercury (30). Treatment of the heme-oxidized peptide with silver did not release any heme, whereas parallel treatment of cytochrome c removed all of its heme (not shown). Thus, the linkage of heme in the heme-binding domain appears not to be via a thioether linkage.

DISCUSSION

The results presented here establish that IRP2 has a heme-binding domain. Studies are currently in progress to determine the three-dimensional structure of the heme-binding domain of IRP2, and we expect to find a cysteine sulfur liganded to the heme because we observed a hyperporphyrin difference spectrum upon the binding of heme to the peptide. We found that Cys-174 and Cys-178 could be oxidatively desulfurated, whereas Cys-168 was not. Conversely, Cys-168 was the primary residue for covalent linkage to heme. Thus, Cys-168 may be axially liganded to heme. After oxygen binds as the other axial ligand, the oxidizing species could attack Cys-174 or Cys-178. Because alanine is a major product, the reaction is that of a cysteine lyase. The known cysteine lyases are pyridoxal phosphate-dependent enzymes (31). Although no cytochrome-dependent cysteine lyases have yet been described, cytochrome P450sd catalyze oxidative desulfurations (32). Cleavage of a carbon-sulfur bond by a thiolute has been demonstrated with low molecular weight alkene thiolates (33). Cysteine lyases yield sulfur as their other product and are important in supplying sulfur for the synthesis of iron-sulfur centers. We tested whether the heme-binding domain could function catalytically as a cysteine lyase but were unable to detect free alanine when incubated with free cysteine (not shown).

Thus, we believe that the domain has evolved as a heme sensor rather than as a cysteine lyase. At a concentration of 10 μM, the heme-binding domain was saturated by 20 μM heme. Although the intracellular concentration of “free” heme is not well established, other heme-regulated systems respond to similar concentrations of heme. Mitochondrial δ-aminolevulinic acid synthase is synthesized in the cytosol with a signal peptide targeting it for import by the mitochondrion. The import is regulated by heme and is −90% inhibited by 25 μM heme (18). The activity of reticulocyte δ-aminolevulinic acid synthase is decreased when the cells are exposed to 40 μM heme, and this is not due to direct inhibition of the enzyme (34).

IRP2 regulates iron metabolism, and IRP2, in turn, is regulated by iron. The analyses presented in this paper establish that the novel IRP2 domain can function as a heme sensor, a physiologically effective mechanism of monitoring cellular iron status. The domain confers upon IRP2 the capability of self-modification; no other proteins are required for covalent modification in the presence of heme and oxygen. What function would be served by this reaction? Does it regulate the proteolytic degradation of IRP2?

We demonstrated previously that oxidative modification of IRP2 renders it a substrate for ubiquitinylation in vitro and
Following oxidative modification by heme, the peptide was alkylated and then digested by trypsin. The mixture was separated by reverse phase chromatography and sequenced by mass spectrometry. The positions of cysteine and alanine are highlighted by boldface type in the sequences.

| Cysteine | Residues | Calculated mass | Observed mass | Difference | Assigned sequence |
|---------|----------|-----------------|--------------|------------|------------------|
| 168     | 166–169  | 544.2792        | 544.2801     | +0.0009    | LPCCR            |
| 174     | 170–175  | 721.3177        | 721.3207     | +0.0100    | QGTTCR           |
| 178     | 176–185  | 1,036.4244      | 1,036.4249   | +0.0005    | GSCDSGELGR       |

Following oxidative modification by heme, the peptide was alkylated and then digested by trypsin. The mixture was separated by reverse phase chromatography and sequenced by mass spectrometry. The positions of cysteine and alanine are highlighted by boldface type in the sequences.

![In-gel assay of heme covalently bound to the heme-binding domain.](image)

**Fig. 4.** In-gel assay of heme covalently bound to the heme-binding domain. Wild type or site-specific mutants (Cys to Ala) were incubated for 2 h at 37 °C in room air at a peptide concentration of 5 μM and a heme concentration of 10 μM. The samples were processed as described under “Experimental Procedures,” allowing heme bound to the peptide to be visualized by its peroxidase activity. Panel A demonstrates that Cys-168 is critical for heme binding. The mutants are designated by their order in the sequence so that C1 is Cys-168, C2 is Cys-174, and C3 is Cys-175. WT, wild type. Panel B shows the pH dependence of the reaction for the wild-type peptide.

also that ubiquitylated IRP2 accumulates in iron-replete cells, provided that the proteasome is inhibited (36). Iwai and colleagues then identified HOIL-1 as a ubiquitin ligase that acts on heme-oxidized IRP2 (35). In vivo, HOIL-1 presumably binds to oxidatively modified IRP2 but not to the native form, although we have not yet been able to demonstrate this assumption in vitro. Following ubiquitylation by HOIL-1, the IRP2 would be degraded by the 26 S proteasome. However, three laboratories have presented convincing evidence that the cysteine-rich heme-binding domain is not required for iron or hemin-triggered degradation of IRP2, and the degradation appears to require 2-oxoglutarate (7–9). An iron, oxygen, and oxoglutarate process triggers degradation of the transcription factor hypoxia-inducible factor-1 through the action of prolyl hydroxylases (36–38). Because the prolyl hydroxylases require iron for activity, one can readily envision a mechanism in which these hydroxylases serve as an iron sensor and mediate the covalent modification of IRP2, just as they function as an oxygen sensor in the hypoxia-inducible factor-1 system.

Given the presence of the heme-binding domain in IRP2 and the existence of HOIL-1 as a ubiquitin ligase acting on heme-oxidized IRP2, we suggest that there exists more than one mechanism for triggering the proteolytic degradation of IRP2. Elucidation of the physiological roles of each pathway and their organ or tissue specificity will naturally require experimental investigation. Both the domain-dependent and domain-independent pathways require oxygen, and it may be illuminating to determine the oxygen concentration dependence of the two pathways, as they may be activated at different partial pressures of oxygen.

The two pathways may also link to different forms of the proteasome, which exhibits heterogeneity in both the 20 and 26 S forms (39). It is known that the 20 S proteasome is capable of distinguishing native from modified proteins; the latter are proteolytically degraded whereas the former are not (40). In particular, oxidatively modified proteins are readily distinguished from native proteins by the 20 S proteasome both in vitro (41–43) and in vivo (44, 45). Investigations from the Grune and Davies laboratories established that ubiquitin is not required for the in vivo degradation of oxidatively modified proteins (46). This finding leaves open for experimental investigation the possibility that oxidatively modified IRP2 can be degraded by both ubiquitin-dependent and ubiquitin-independent pathways (39).

The thiolate-ligated cytochrome P450s have long been known to self-inactivate through auto-oxidation (47). We suggest that the auto-oxidation of the thiolate-ligated heme-binding domain provides an elegantly simple mechanism for regulation of the turnover of IRP2, although this is not an exclusive mechanism.

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**REFERENCES**

1. Rouault, T., and Klausner, R. (1997) *Curr. Top. Cell. Regul.* 35, 1–19
2. Hentze, M. W., and Kuhn, L. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 8175–8182
3. Binder, R., Horowitz, J. A., Basilion, J. P., Koeller, D. M., Klausner, R. D., and Harford, J. B. (1994) *EMBO J.* 13, 1869–1880
4. Guo, B., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) *J. Biol. Chem.* 270, 21645–21651
5. Iwai, K., Klausner, R. D., and Rouault, T. A. (1995) *EMBO J.* 14, 5350–5357
6. Iwai, K., Drake, S. K., Wehr, N. B., Weissman, A. M., LaVaute, T., Minta, N., Klausner, R. D., Levine, R. L., and Rouault, T. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 4924–4928
7. Hanson, E. S., Rawlins, M. L., and Leibold, E. A. (2003) *J. Biol. Chem.* 278, 40337–40342
8. Bourdon, E., Kang, D. K., Ghosh, M. C., Drake, S. K., Wey, J., Levine, R. L., and Rouault, T. A. (2003) *Blood Cells Mol. Dis.* 31, 247–255
9. Wang, J., Chen, G., Muckenthaler, M., Galy, B., Hentze, M. W., and Pante polous, K. (2004) *Cell Mol. Biol.* 40, 854–865
10. Kim, S., Wing, S. S., and Ponka, P. (2004) *Cell Mol. Biol.* 40, 330–337
11. Kang, D. K., Jeong, J., Drake, S. K., Wehr, N. B., Rouault, T. A., and Levine, R. L. (2003) *J. Biol. Chem.* 278, 14857–14864
12. O'Halloran, T. V., and Culotta, V. C. (2000) *J. Biol. Chem.* 275, 25057–25060
13. Aisen, P., Wesselingh-Resnick, M., and Leibold, E. A. (1999) *Curr. Opin. Chem. Biol.* 3, 209–206
14. Drabkin, D. L. (1951) *Physiol. Rev.* 31, 435–431
15. Goessling, L. S., Mascotti, D. P., and Thach, R. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 13056–13061
16. Yamanaka, K., Ishikawa, H., Megumi, Y., Tokunaga, F., Kanie, M., Rouault, T. A., Morishima, I., Minato, N., Ishimori, K., and Iwai, K. (2003) *Nat. Cell Biol.* 5, 336–340
17. Qi, Z., Hamza, I., and O'Brian, M. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 3753–3757
18. Lathrop, J. T., and Timko, P. M. (1993) *Science* 259, 522–525
19. Vuletic, J. L., and Osawa, Y. (1998) *Anal. Biochem.* 265, 375–380
20. Hanson, L. K., Eaton, W. A., Sliger, S. G., Gunsalus, I. C., Gouterman, M., and Connell, C. R. (1976) *J. Am. Chem. Soc.* 98, 2672–2674
21. Collman, J. P., Sorrell, T. N., Dawson, J. H., Trudell, J. R., Bunnenberg, E., and Djerassi, C. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 6–10
22. Sono, M., Anderson, L. A., and Dawson, J. H. (1980) *J. Biol. Chem.* 257,
23. Gouterman, M. (1978) in *The Porphyrins*, (Dolphin, D., ed) Vol. III, pp. 1–165, Academic Press, New York.
24. Ruf, H. H., and Wende, P. (1977) *J. Amer. Chem. Soc.* 99, 5499–5500.
25. Chang, C. K., and Dolphin, D. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 3338–3342.
26. Sono, M., Dawson, J. H., and Hager, L. P. (1984) *J. Biol. Chem.* 259, 13209–13216.
27. Inada, Y., and Shibata, K. (1962) *Biochem. Biophys. Res. Commun.* 9, 323–327.
28. Mansuy, D., Duppe, W., Ruf, H. H., and Ulrich, V. (1974) Hoppe-Seyler's *Z. Physiol. Chem.* 355, 1341–1349.
29. Osawa, Y., and Korzekwa, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 7081–7085.
30. Williams, V. P., and Glazer, A. N. (1978) *J. Biol. Chem.* 253, 202–211.
31. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) *Biochemistry* 33, 4714–4720.
32. Grune, T., Reinheckel, T., and Davies, K. J. (1996) *Biochemistry* 415, 1–5.
33. Grune, T., Reinheckel, T., and Davies, K. J. (1996) *Biochemistry* 278, 26–34.
34. Davies, K. J., Lin, S. W., and Pacifici, R. E. (1987) *J. Biol. Chem.* 262, 9914–9920.
35. Yamanaka, K., Ishikawa, H., Megumi, Y., Tokunaga, F., Kanie, M., Rosault, T. A., Morishima, I., Minato, N., Ishimori, K., and Iwai, K. (2003) *Nat. Cell Biol.* 5, 336–340.
36. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) *Science* 292, 464–468.
37. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schufield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) *Science* 292, 468–472.
38. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) *EMBO J.* 20, 5197–5206.
39. Orlowksi, M., and Wilk, S. (2003) *Arch. Biochem. Biophys.* 415, 1–5.
40. Rivett, A. J. (1986) *Curr. Top. Cell. Regul.* 28, 291–337.
41. Rivett, A. J. (1985) *Arch. Biochem. Biophys.* 243, 624–632.
42. Rivett, A. J., and Levine, R. L. (1990) *Arch. Biochem. Biophys.* 278, 26–34.
43. Davies, K. J., Lin, S. W., and Pacifici, R. E. (1987) *J. Biol. Chem.* 262, 9914–9920.
44. Grune, T., Reinheckel, T., and Davies, K. J. A. (1996) *J. Biol. Chem.* 271, 15504–15509.
45. Grune, T., Merker, K., Sandig, G., and Davies, K. J. (2003) *Biochem. Biophys. Res. Commun.* 305, 709–718.
46. Shringarpure, R., Grune, T., Mehlhase, J., and Davies, K. J. (2003) *J. Biol. Chem.* 278, 311–318.
47. Sligar, S. G., Lipscomb, J. D., Debrunner, P. G., and Gunsalus, I. C. (1974) *Biochem. Biophys. Res. Commun.* 61, 290–296.
