Human Biliverdin Reductase Is Autophosphorylated, and Phosphorylation Is Required for Bilirubin Formation*

Mohammad Salim, Brigette A. Brown-Kipphut, and Mahin D. Maines‡

From the Department of Biochemistry/Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

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Biliverdin reductase (BVR) reduces heme oxygenase (HO) activity product, biliverdin, to bilirubin. BVR is unique in having dual pH/dual cofactor requirements. Using Escherichia coli-expressed human BVR and COS cells, we show that BVR is autophosphorylated and that phosphorylation is required for its activity. An “in blot” autophosphorylation assay showed that BVR is a re- naturable phosphoprotein. Controls for the experiments were HO-1 and HO-2; both are phosphoproteins but are not autophosphorylated. Autophosphorylation was pH-dependent, with activity at pH 8.7 being most prominent. In addition, 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate fluorescence titration of BVR gave a lower $K_m$ at pH 8.7 than at pH 7.4 (15.5 versus 28.0 μM). Mn$^{2+}$ was required for binding of the ATP analog and for autophosphorylation; the autokinase activity was lost when treated at 60 °C for 10 min. The loss of transferred phosphates by alkaline treatment suggested that BVR is a serine/threonine kinase. Potato acid phosphatase treatment reversibly inactivated the enzyme. The enzyme was also inactivated by treatment with the serine/threonine phosphatase, protein phosphatase 2A; okadaic acid attenuated the inhibition. Titration of protein phosphatase 2A-released phosphates indicated a 1:6 molar ratio of BVR to phosphate. The BVR immunoprecipitated from COS cell lysates was a phosphoprotein, and its activity and phosphorylation levels increased in response to H$_2$O$_2$. The results define a previously unknown mechanism for regulation of BVR activity and are discussed in the context of their relevance to heme metabolism.

In the cell, the negative feedback inhibition of heme oxygenase (HO) activity by biliverdin is a mechanism by which oxidation of heme is regulated (1, 2). Biliverdin is the open tetrapyrole oxidation product of heme (iron-protoporphyrin IX, hemin, heme b) degradation by the HO system, also called the HSP32 family of proteins (1, 3–5). In various species including mammals and certain fish, biliverdin undergoes reduction by the oxidoreductase biliverdin reductase (BVR) (6–11); the reductase catalyzes the reduction of the γ-meso bridge of biliverdin to bilirubin. Synthesis of biliverdin is a prominent dimension of HO system function in cellular defense mechanisms (12).

In plants, biliverdin analogues function in a photoregulatory capacity (13–16), and in cyanobacteria BVR-catalyzed conversion of biliverdin to bilirubin is important for normal phycoliprotein synthesis (17). In mammals, bilirubin has antioxidant and immunomodulatory properties (18–28), induces Aryl hydrocarbon receptor-mediated activation of cytochrome P450, and antibody-dependent and -independent cell-mediated cytotoxicity (21–28). Interestingly, there is a report suggesting that higher serum bilirubin levels are associated with decreased risk for early familial coronary artery disease (24).

BVR is unique among all enzymes characterized to date; previous studies have identified the reductase as the only dual pH/dual adenine nucleotide cofactor-requiring enzyme (9, 32–35). The reductase, which is highly conserved in its primary structure and molecular properties, uses NADPH in the acidic range (peak activity at pH ~6.7), whereas NADP$^+$ is utilized in the basic range (peak activity at pH ~8.7). BVR is encoded by a single copy gene (36) and is posttranslationally modified (32).

This study identifies, for the first time, BVR as a phosphoprotein and finds that phosphorylation is essential to the activity of BVR to reduce biliverdin to bilirubin. Furthermore, it is found that the enzyme is autophosphorylated and that phosphorylation is reversible; the latter criterion defines a kinase. Based on the findings, we suggest that reversible phosphorylation of BVR is a previously unknown mechanism by which biological activities of heme degradation products are regulated in the cell.

MATERIALS AND METHODS

Chemicals—Cofactors, α-casein, and biliverdin-HCl were purchased from Sigma or Porphyrin Products (Logan, UT). Mixtures of anti-phosphoserine and anti-phosphothreonine or of anti-phosphoserine, anti-phosphothreonine, and anti- phospho-tyrosine were obtained from Promega. The sources of other reagents are noted in connection with the appropriate experiments. All chemicals used were of the highest purity commercially available.

Purified Enzyme Preparations—BVR was purified from human liver to homogeneity as before (32, 37) and was used for preparation of a polyclonal antibody in New Zealand rabbits (9). Human HO-1 and HO-2 were expressed in Escherichia coli and purified as before (38).

Purification of E. coli-expressed Human BVR and Measurement of Activity—To generate glutathione S-transferase (GST)-BVR fusion protein, wild type human BVR (34) plasmid was used as a template for polymerase chain reaction using the primers GGTCGACGAATGCA-GTGCAATTGAATGTCAGTTACTCTTG (italicized), respectively, and containing SaII linkers (underlined). The product was digested with SaII and cloned into the vector pGEX-4T-2. Orientation was determined by restriction analysis and confirmed by sequencing. The ligation places the BVR coding region in-frame with the GST protein of the vector.

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‡ To whom correspondence should be addressed. Tel.: 716-275-5383; Fax: 716-275-6007; E-mail: mahin.maines@urmc.rochester.edu.

The abbreviations used are: HO, heme oxygenase; BVR, biliverdin reductase; GST, glutathione S-transferase; MBP, myelin basic protein; TNP-ATP, 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; PP2A, protein phosphatase 2A; TES, 2-(hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulfonic acid.

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GST-BVR fusion protein was purified from bacterial clones containing the plasmid and grown overnight, using a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). The reductase portion of the fusion protein was released by thrombin protease treatment. Activity was determined as previously described (9, 32). The rate of activity was measured in the presence of 5 mM NADH at 37 °C. Specific activity is expressed as nmol of bilirubin/min/mg of protein.

**Phosphotransferase Activity**—The phosphotransferase activity of the reductase was examined using two different methods: “in blot” and “in solution” kinase assays. The in blot kinase assay was based on the procedure described by Ferrell and Martin (39). Following electrophoresis, the SDS-polyacrylamide gel electrophoresis protein bands were transferred to nitrocellulose membrane (Millipore). The blot was denatured for 1 h at room temperature in 50 mM Tris-HCl, pH 8.3, 7 mM guanidine-HCl, 50 mM dithiothreitol, and 2 mM EDTA. The bound protein was allowed to renature in 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, 1% bovine serum albumin, 0.1% Nonidet P-40 overnight at 4 °C. The blot was treated with 5% bovine serum albumin in 30 mM Tris-HCl at pH 7.4 at room temperature for 1 h. It was then equilibrated in 30 mM Tris-HCl of appropriate pH and subjected to the [γ-32P]ATP labeling mixing, consisting of 30 mM Tris (of appropriate pH), 10 mM MgCl2, 2 mM MnCl2, 30 μM ATP, and ~400 μCi/ml [γ-32P]ATP for 40 min at room temperature. The blot was then washed with 250 μl of the following: three times in 30 mM Tris-HCl, pH 8.3 and 7.4, incubated in appropriate experiments, one time in 1% SDS buffer containing 0.5% Nonidet P-40, 4 times in buffer without Nonidet P-40, once in 1 M KOH, and two times with Tris buffer only. The membrane was dried and exposed for autoradiography.

The in solution kinase assay protocol of Brown et al. (40) was used at pH values indicated in appropriate experiments. The reaction mix contained 30 mM Tris-HCl at the appropriate pH, 0.5 mM dithiothreitol, 10 mM MgCl2, 30 μM ATP (Sigma), and 5 μCi of [γ-32P]ATP/50 μl. The metal dependence of BVR kinase activity was measured in the presence of 0, 1, and 2 mM concentrations of Ca2+ or Mn2+. The reaction contained 5 μl of purified BVR. When used, the reaction also contained 10 μl of dephosphorylated α-casein (Sigma) or myelin basic protein (MBP, Sigma). The assay mix was incubated at 37 °C for 1 h and was terminated by adding 10 μl of 1% SDS. Unincorporated ATP was removed by gel filtration through a Sephacryl G-50 column (Amersham Pharmacia Biotech), and the eluant was loaded onto a 0.75-mm, 15% SDS-polyacrylamide gel (41). The gel was stained using Coomassie Blue and dried under a vacuum. The same gel was then exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) and developed.

To determine whether BVR serine/threonine residues are a target of autophosphorylation, the following experiment based on methodology described by Kamps and Sefton (42) was carried out. BVR (20 μg) was subjected to in solution phosphorylation. After removal of unbound ATP and treatment with SDS at 100 °C, samples were applied to gel and transferred to an Immobilon P membrane. The membrane was briefly exposed for autoradiography. The same membrane was subsequently cut in two. One section was treated with 1% KOH for 2 h at 55 °C to hydrolyze phosphates from phosphoserine and phosphothreonine residues. The second section was treated similarly but at pH 7.4. The membrane was again autoradiographed.

**ATP Binding**—The pH dependence of BVR affinity for ATP was determined by measuring the increase in fluorescence after addition of increasing amounts of the ATP analogue TNP-ATP to BVR as described by Senior and colleagues (43, 44). Fluorescence measurements were made in a SPEX Fluorolog 2 fluorometer. 2-ml stirred cuvettes at room temperature containing 30 mM Tris-HCl (pH 6.7, 7.4, and 8.7), 2 mM Mn2+, 10 mM Mg2+, and 1.0 μM BVR were used for TNP-ATP binding experiments. The excitation wavelength was 408 nm, and the emission spectra from 500 to 600 nm were recorded. A control cuvette contained all components except BVR. Fluorescence spectra were taken 1 min after the addition of TNP-ATP. The TNP-ATP concentration was determined using an extinction coefficient of 26,400 M⁻¹ cm⁻¹ at 408 nm.

**Dephosphorylation of BVR**—The enzyme was dephosphorylated using two different enzymes: potato acid phosphatase and protein phosphatase 2A (PP2A). For the former, E. coli containing BVR plasmid construct was grown overnight in LB medium. The cells were sonicated in phosphate-buffered saline (10 mM phosphate buffer, pH 7.4, 140 mM NaCl) and centrifuged at 10,000 × g for 15 min. GST-BVR fusion protein in the cell extract was then bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C for 1 h. Beads were washed four times with phosphate-buffered saline and then four times with TES buffer (100 mM TES, pH 7.0, 100 mM NaCl). Beads were incubated at 37 °C for 2 h with 15 units of potato acid phosphatase (Sigma) in TES buffer to remove phosphate. After incubation, beads were washed six times with TES buffer to remove phosphate. BVR was then cleaved off GST fusion by incubation with 5 units of thrombin protease at 4 °C overnight. The protein solution was collected and measured by a Bradford assay. To rephosphorylate BVR, the assay conditions described above for the in solution kinase assay were used.

That phosphorylation is required for its reductase activity was also examined, and the number of phosphates bound to BVR was determined after PP2A (Upstate Biochem) treatment. BVR (8 μg) was treated with PP2A (1.0 unit) and incubated for 30 min at 30 °C. To test for specificity of phosphatase activity, PP2A was inhibited by okadaic acid (1 μM) by incubating 30 °C for 5 min with prior to addition of BVR. Phosphatase activity was measured at pH 6.7 with NADH as the cofactor. To measure the stoichiometry of BVR-phosphate after treatment with PP2A, the released phosphate was quantitated based on a phosphate standard titration curve constructed using EnzChek phosphate assay kit (E-66464, Molecular Probes).

**Cell Culture**—COS cells were grown to 80% confluency in Dulbecco’s medium containing 10% fetal calf serum. The cells were suspended in prewarmed serum-free Dulbecco’s modified Eagle’s medium at 37 °C. Cell suspension was divided into aliquots. H2O2 (1 mM) was added to the cell suspensions, and they were incubated at 37 °C. Control samples were not treated. After centrifugation at 2000 rpm for 5 min, cells were lysed in 50 μl of lysis buffer A (50 mM Tris-HCl, pH 7.4, 75 mM NaCl, 1% Nonidet P-40, 0.2 mM Na3VO4, 10 mM NaF, 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each leupeptin, pepstatin, and aprotinin, 2 mM EDTA, 2 mM EGTA) on ice for 15 min. After a brief sonication, the lysates were centrifuged at first at 1000 rpm for 5 min, and then the supernatant fraction was centrifuged at 45,000 rpm for 60 min. Protein concentration was measured by Bradford assay (Bio-Rad).

For BVR immunoprecipitation, cells were lysed in lysis buffer as above. Anti-BVR antibody coupled to protein A was added to the supernatant fraction. For coupling, 10 μl of antibody was incubated with 40 μl of protein A-Sepharose, which had been blocked with bovine serum albumin for 3 h at 4 °C. 100 μg of protein of cell lysate was added to beads, and the mixture was incubated for a minimum of 3 h at 4 °C. The immunoprecipitate was recovered by centrifugation, washed three times with SDS-sample buffer, and used for SDS-polyacrylamide gel electrophoresis and immunoblotting.

Duplicate blots were probed with anti-BVR and antiphosphomix consisting of a mix of anti-serine/anti-threonine phospho-amino acids.

**RESULTS**

Previous studies have suggested that BVR is posttranslationally modified (32). In this study, experiments were carried out to determine whether BVR is a phosphoprotein and, if so, whether phosphorylation of BVR is a reflection of autophosphorylation activity. Autophosphorylation activity was analyzed by two different methods: the in blot and in solution kinase assays. The results of the in blot kinase assay are shown in Fig. 1A. Autophosphorylation activity was assessed, after denaturation of BVR with guanidine-HCl, from phosphate incorporation into the renatured protein in the presence of [γ-32P]ATP. Incorporation of [32P]PO4 upon renaturation was detected by autoradiography. As shown when renatured in the presence of ATP, phosphorylated BVR was transferred to BVR. HO-1 and HO-2, which are phosphoproteins, as indicated by immunoreactivity toward a mixture of anti-serine/anti-threonine/anti-tyrosine (Fig. 1B), were used as controls for autophosphorylation. As noted in Fig. 1A, neither of the HO isozymes is reversibly phosphorylated under the same conditions that showed incorporation of the γ phosphate of ATP into BVR. The finding suggested that the labeled phosphate signal produced by renatured BVR was not due to nonspecific protein binding of [γ-32P]ATP.

Because of the pH dependence of BVR, the effect of pH on phosphotransferase activity was measured at pH 8.7 and 6.7. In addition, experiments were performed to examine whether the γ 32P signal observed in Fig. 1A is an authentic reflection of its incorporation in BVR in the process of renaturation, rather than a reflection of nonspecific protein binding. For these experiments, the in solution kinase assay was used. The results are shown in Fig. 1C. Casein, which is not a phosphoprotein
Biliverdin Reductase Kinase Activity

**Fig. 1.** Biliverdin reductase is autophosphorylated. A, the in solution kinase assay. 20-μg aliquots of purified human or E. coli-expressed human BVR, HO-1, or HO-2 preparations, after SDS-gel electrophoresis, were transferred to a polyvinylidene difluoride membrane and examined for renaturable phosphorylation at pH 7.4. The protein was denatured, renatured, and incubated in the presence of 32P-labeled ATP as detailed under “Materials and Methods.” B, 4 μg each of the same enzyme preparations were subjected to Western blot analysis using anti-phospho mix as the primary antibody. The ECL method of detection was used. C, the in solution kinase assay. The kinase assay was carried out in the presence of 2 μM Mn2+ and 10 μg of dephosphorylated α-casein. 5 μg of purified E. coli-expressed BVR was used for each assay (50 μl). Phosphotransferase activity was carried out at the indicated pH values. The gel was processed as detailed under “Materials and Methods.” D, the same gel stained with Coomassie Blue before autoradiography.

and is a commonly used acceptor of phosphate, was used in these experiments as a negative control for autophosphorylation. The stained gel of the same samples for control of sample loading is shown in Fig. 1D. As noted in Fig. 1C, BVR auto-phosphorylation is pH-dependent, with the activity being prominent at pH 8.7 and essentially no phosphotransferase activity occurring at pH 6.7. Moreover, only in lanes that contained BVR was the 32P signal observed. In lanes that contained casein alone, a signal was not observed under the pH conditions. This observation suggested that the autophosphorylation of BVR in this kinase system also is not a reflection of nontargeted ATP binding to the protein. Moreover, it appeared that at pH 8.7 BVR transferred phosphate to casein.

To confirm that BVR transfers phosphate to casein in the in solution kinase assay was carried out in the presence of increasing concentrations of casein (1–20 μg) and a constant concentration of BVR (5 μg). The data suggest that BVR does transfer phosphate to casein, albeit not at an impressive rate, as indicated by the kinetics of the reaction (K_m = 1.1 μM; V_max = 2.8 pmol/mg/min). It is noted that the pattern of casein separation on SDS gel reveals that the commercially obtained casein separates on the gel as multiples of protein bands. When compared with Fig. 1C, it is evident that not all components that are present in the casein preparation are phosphorylated in the presence of BVR.

Next, the effect of pH on ATP binding was examined using the fluorescent ATP analogue TNP-ATP. The increase in fluorescence of TNP-ATP as the result of binding to BVR was measured. The binding was titrated as an increase in BVR fluorescence at 550 nm as a function of TNP-ATP concentration. As shown in Fig. 3, at pH 7.4 and 8.7 there is a TNP-ATP fluorescence at 550 nm as a function of TNP-ATP concentration. The fluorescent ATP analogue TNP-ATP was used in each assay. Assays were also carried out in the presence of MBP (10 μg). A, lanes: 1, BVR, 2, BVR plus MBP, 3, MBP. B, lanes: 1, MBP; 2, BVR plus MBP; 3, BVR. C, lanes: 1, BVR heated for 10 min at 60 °C; 2, the same preparation without heating. Top panel, in solution kinase activity at pH 8.7; bottom panel, the same gel stained with Coomassie Blue.

**Fig. 2.** Characterization of phosphotransferase activity of human biliverdin reductase. Purified E. coli-expressed BVR was analyzed by an in solution kinase assay in the presence (A) or absence (B) of 2 mM MnCl2 at pH 8.7 as described under “Materials and Methods.” 5 μg of BVR was used in each assay. Assays were also carried out in the presence of MBP (10 μg). A, lanes: 1, BVR, 2, BVR plus MBP, 3, MBP. B, lanes: 1, MBP; 2, BVR plus MBP; 3, BVR. C, lanes: 1, BVR heated for 10 min at 60 °C; 2, the same preparation without heating. Top panel, in solution kinase activity at pH 8.7; bottom panel, the same gel stained with Coomassie Blue.

The phosphotransferase activity of BVR specifically required Mn2+ (Fig. 2). Ca2+ used at the same concentrations as Mn2+ (1.0 and 2.0 mM) did not support phosphotransferase activity. As shown in Fig. 2, A versus B, at pH 8.7 in the absence of Mn2+, no incorporation of labeled phosphate in BVR takes place (panel A, lane 1 versus panel B, lane 3). The Mn2+ requirement for BVR phosphotransferase activity was further examined in an assay system containing MBP. As detected on the autoradiograph (Fig. 2A, lane 2), MBP, a phosphate acceptor, when present with BVR plus Mn2+, also gave three distinct 32P signals. In the absence of Mn2+ the signals were quite subdued (Fig. 2B, lane 2). MBP is known to nonspecifically bind phosphate in the absence of a kinase, and this was also observed in our hands (Fig. 2A, lane 3); as noted in panel B, lane 1, this interaction can take place in the absence of Mn2+. Commercially available MBP, as is the case with casein, separates in several bands on SDS gel. Because the objective of this experiment was not to characterize MBP phosphorylation, the kinase activity of BVR with MBP was not further examined. The autophosphorylation activity of BVR was thermostable and was lost by exposure to 60 °C for 10 min prior to use in the kinase assay (Fig. 2C, lane 1).
Biliverdin Reductase Kinase Activity

Fig. 3. Binding of TNP-ATP to human BVR. Purified E. coli-expressed BVR was titrated with TNP-ATP in an assay system containing 30 mM Tris buffer at pH 7.4 or 8.7, 10 mM Mg²⁺, 2 mM Mn²⁺, and the indicated concentrations of TNP-ATP. The BVR concentration in the assay system was 1 µM. The increase in fluorescence was measured at 550 nm. ○—○, pH 8.7; ●—●, pH 7.4.

Amino residues, as suggested by results of the experiments shown in Fig. 4. Subsequent to autophosphorylation and autoradiography (Fig. 4A) (control for protein loading), the membrane was divided in two parts; one part was treated with 1 N KOH (2 h at 55 °C) and again autoradiographed. As shown in Fig. 4B, the intensity of the ³²P signal is reduced to a minimum subsequent to alkaline hydrolysis (lane 1 versus lanes 2 and 3). Fig. 4A shows that the decrease in intensity is not due to a differential loading of the lanes (lane 1 versus lanes 2 and 3).

The significance of the phosphorylation state to reductase activity was examined by investigating the effect of treatment with phosphatases on BVR activity. The first experiment used potato acid phosphatase. For this experiment, a protocol was designed to ensure the removal of the added phosphatase from the BVR preparation, and only NADH-dependent activity was measured, to ensure that NADPH dephosphorylation by phosphatase did not contribute to the observation. The results are shown in Table I. As noted, treatment with potato acid phosphatase caused a dramatic decrease in BVR activity. The loss of activity was, however, recovered when the phosphatase-treated preparation was incubated with ATP at pH 8.7 under the same conditions used for in solution autophosphorylation and electrophoresis on a 12% SDS-polyacrylamide gel and then transferred to an Immobilon membrane. The membrane was autoradiographed for control of loading. Subsequently, the membrane was cut in two, and one section was subjected to alkaline hydrolysis (1 N KOH) before autoradiography. Experimental details are provided under “Materials and Methods.” A, lane 1, ³²P signal before hydrolysis; B, lane 1, ³²P signal after hydrolysis. A and B, lanes 2 and 3, control.

Table I

| Sample                  | ATP (50 µM) | Activity % |
|-------------------------|-------------|------------|
| Control BVR             | –           | 100        |
| BVR + phosphatase       | +           | 139        |
| BVR + phosphatase       | –           | 17         |
| BVR + phosphatase       | +           | 151        |

Next, we examined whether phosphorylation of BVR also occurs in the cell and whether it has a bearing on BVR activity. For these experiments BVR was immunoprecipitated from COS cells and analyzed for phosphorylation using a mixture of anti-phosphoserine and anti-phosphothreonine antibodies. The results are shown in Fig. 6. As noted in Fig. 6A (top panel), BVR is phosphorylated in vivo (lane 1), and its phosphorylation is increased in a time-dependent manner in response to H₂O₂ treatment. BVR phosphorylation did not increase further when...
and response to H$_2$O$_2$. COS cells were treated with 1 mM H$_2$O$_2$. Control cells were not treated. At the indicated times, cells were lysed, and BVR was immunoprecipitated and subjected to electrophoresis and blot transfer. For one blot, a mix of anti-phosphoserine and anti-phosphothreonine was used as the primary antibody (top panel). Anti-BVR antibody was used for the second blot (bottom panel). BVR was immunoprecipitated and subjected to electrophoresis and analysis under “Materials and Methods.” The activity analysis was repeated using two separate preparations of COS cells.

assessed after 1 h of H$_2$O$_2$ treatment. The increased phosphorylation was not a reflection of an increase in cellular BVR protein levels, as suggested by the Western blot analysis using anti-BVR antibody (Fig. 6A, bottom panel). The reductase activity was also increased in cells treated with H$_2$O$_2$ (Fig. 6B). As shown, a time-dependent increase in activity was detected in the presence of H$_2$O$_2$, with the rate of bilirubin formation measuring 3–5-fold that of the control when determined at 15 and 60 min, respectively, after treatment with H$_2$O$_2$. The early onset of increasing activity suggests that activation of the protein, rather than increased BVR protein, is responsible for the observation.

**DISCUSSION**

This report has identified phosphotransfer and reduction of biliverdin as two types of activities of human BVR and has shown that the reductase activity of the enzyme requires phosphorylation of the protein. Moreover, as with reductase activity, the enzyme shows pH dependence for autophosphorylation. The enzyme appears to be best suited for both autokinase (Fig. 1 and 2) and reductase activity (9) in an alkaline pH range (7.4 and 8.7). pH 6.7 corresponds to the pH value for peak reductase activity in the acidic range, whereas at this pH, phosphotransferase activity was nearly undetectable (Fig. 1C). A plausible explanation for the observation is a decreased affinity of BVR for adenine nucleotides in the acidic range. This possibility is consistent with BVR activity at pH 6.7 requiring a 10-fold higher concentration of cofactor (9, 33, 34) and with the near doubling of the $K_v$ value for TNP-ATP at pH 7.4 versus pH 8.7 (Fig. 3). The base-line fluorescence of TNP-ATP is prohibitive of using a great excess of the agent in the assay system. The fact that at pH 8.7 the enzyme is an NADPH-dependent reductase and is also most effective in autophosphorylation would suggest that at this pH the electronic environment of the phosphate-interacting domains and/or the structure that BVR assumes at this pH allow for high affinity binding of adenine nucleotides. The biological significance of why BVR is a more effective reductase and autokinase at pH 8.7 than at pH 6.7 is not understood.

Hydrolysis of BVR-bound phosphates under alkaline conditions suggests that BVR is a serine/threonine phosphoprotein. Phosphorylated tyrosine residues are resistant to alkaline/acid hydrolysis (42). Findings with PP2A, which is a serine/threo-
nine phosphatase, supports this conclusion. PP2A studies also suggest a 1:6 molar ratio of BVR-phosphate. At this time it is not known how many of the phosphates are transferred to BVR by autophosphorylation. Also not known is which serine/threonine residues are phosphorylated. Human BVR has 24 serine and 7 threonine residues (34). These inquiries are the subject of our future studies.

The finding with casein phosphorylation is also consistent with the possibility that BVR is a serine/threonine kinase. Casein, as well as MBP, is phosphorylated on serine/threonine residues. It is noteworthy that a predicted BVR sequence, Gly$^{148}$-Ser-Leu-Arg-Phe-Thr-Ala-Ser-Pro, closely resembles the serine/threonine kinase consensus motif Gly-(Thr/Ser)-X-X-(Tyr/Phc)-X-Ala-Pro-Glu (45). It is relevant to note that mutation of the Ser$^{149}$ residue in BVR inactivates the enzyme (data not shown). This finding is consistent with the likelihood that the Gly$^{148}$-Ser-Leu-Arg-Phe-Thr-Ala-Ser-Pro motif in BVR is the serine/threonine kinase domain and that phosphorylation of Ser$^{149}$ is essential for BVR activity. The data further suggest that the other five titratable phosphates released by PP2A treatment are bound to serine/threonine residues, which are not acceptors of BVR phosphotransferase activity, and that they may not be critical for BVR activity. In addition, present in BVR is the Walker homology A motif, Gly$^{15}$-X-Gly-X-Gly, which is conserved in the cyclic nucleotide-regulated protein kinase family. Mutation of the Gly$^{17}$ residue in this motif also inactivates the reductase (data not shown).

The presently described in vitro phosphotransferase activity of BVR, together with the finding with COS cells, although suggestive of BVR having phosphotransferase activity in vivo, does not prove this activity. However, the assay systems used for determining autophosphorylation of E. coli-expressed BVR convincingly suggest that in vitro transfer of ATP phosphate to the reductase is carried out by BVR itself. In the mammalian cell, this may not be the case, and other kinases could participate in phosphorylation of BVR. Accordingly, the finding that in COS cells there is an increase in the phosphorylation state of BVR in response to H$_2$O$_2$ does not necessarily implicate autophosphorylation activity; however, an increase in BVR reductase activity is consistent with the conclusion that phosphorylation is required for BVR reductase activity. Moreover, potato acid phosphatase inactivation of the reductase activity (Table I) and reversible activation of the phosphatase-treated BVR further indicate that phosphorylation is important to BVR enzyme activity. Furthermore, it is reasonable to speculate that in the cell, the phosphotransferase activity of BVR may not be directed only toward itself but also toward other substrates.

Enzyme phosphorylation plays a regulatory role in intermediary metabolism, and many enzymes are phosphoproteins; of these a small fraction are reversibly autophosphorylated. The biological significance of BVR phosphotransferase activity and the dependence of the activity on phosphorylation may be related to the central role of the HO system in cellular defense mechanisms, cell signaling (12, 46), and the complex interactions that exist between the enzymes of the heme degradation pathway and products of heme degradation activity. To elaborate, as noted above, the BVR catalytic activity product bilirubin inhibits protein phosphorylation (29); bilirubin is also a feedback inhibitor of reductase (9). On the other hand, biliverdin is an inhibitor of HO activity (2). In fact, with the exception of biliverdin, to date, no other heme pathway products or normal cell constituents have been found to inhibit HO activity in vivo. In addition, as noted above, induction of HO-1 and generation of bilirubin is suspected to be a significant component of the cellular antioxidant defense mechanisms. The finding that H$_2$O$_2$, which is a potent oxidant and inducer of HO-1 (47),...
causes rapid increases in the activity of the reductase and in its phosphorylation state could be particularly relevant to the control of cellular levels of heme degradation products. In this scenario, the rapid conversion of biliverdin to bilirubin would allow for the derepression of HO activity. Product inhibition of BVR activity then would allow for an increase in effective biliverdin levels and hence permit a return to normal of the HO activity. The finding that the reductase is reversibly phosphorylated is further supportive of the potential significance of BVR phosphorylation to cellular defense mechanisms as demand arises.

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